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STUDIES ON  
THE STRUCTURE AND FUNCTION  
OF GLUTATHIONE SYNTHETASE  
FROM *ESCHERICHIA COLI* B

HIROAKI KATO

1988

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1. The first part of the paper is devoted to a general discussion of the problem of the existence of solutions of the system of equations (1) for arbitrary values of the parameters  $\alpha$  and  $\beta$ . It is shown that for arbitrary values of the parameters  $\alpha$  and  $\beta$  the system of equations (1) has solutions in the form of a series in powers of  $\alpha$  and  $\beta$ .

**To K. I.**

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## ABBREVIATIONS

DEAE	diethylaminoethyl
$D_{max}$	maximum particle dimension
DTNB	5,5'-dithiobis(2-nitrobenzoate)
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetate
$\gamma$ -Glu-Abu	$\gamma$ -L-glutamyl-L- $\alpha$ -aminobutyrate
$\gamma$ -Glu-Cys	$\gamma$ -L-glutamyl-L-cysteine
<i>gshII</i>	glutathione synthetase gene from <i>Escherichia coli</i> B
IPTG	isopropyl $\beta$ ,D-thiogalactopyranoside
$K_i$	inhibition constant
$K_m$	Michaelis constant
<i>L. casei</i>	<i>Lactobacillus casei</i>
LB medium	Luria-Bertani medium
MTX	methotrexate
<i>N. gonorrhoeae</i>	<i>Neisseria gonorrhoeae</i>
NEM	N-ethylmaleimide
NTCB	2-nitrothiocyanobenzoate
pCMB	p-chloromercuribenzoate
Pi	inorganic phosphate
$R_g$	radius of gyration
SAXS	small angle X-ray scattering
SD sequence	Shine-Dalgarno sequence
SDS	sodium dodecyl sulfate
TNB <sup>2-</sup>	2-nitro-5-thiobenzoate anion
Tris	tris(hydroxymethyl)aminomethane
$V_M$	ratio of the volume of the unit cell to the molecular weight of protein in the cell

## GENERAL INTRODUCTION

Glutathione synthetase (EC 6.3.2.3,  $\gamma$ -L-glutamyl-L-cysteine : glycine ligase(ADP-forming)) catalyzes the synthesis of glutathione from  $\gamma$ -Glu-Cys and Gly in the presence of ATP (1,2). Namely, this enzyme catalyzes the second reaction in the glutathione biosynthetic pathway in which firstly  $\gamma$ -Glu-Cys is synthesized by  $\gamma$ -glutamylcysteine synthetase (EC 6.3.2.2, L-glutamate : L-cysteine  $\gamma$ -ligase(ADP-forming)) (3,4).

Glutathione synthetase is distributed in a wide variety of sources (1,2). Highly purified enzyme preparations have been obtained from yeast (5), rat kidney (6), and *Escherichia coli* B (7). Molecular weight of these enzymes are determined as 12,3000 for the yeast, 118,000 for the rat kidney, and 142,000 for the *E. coli* B preparations. An optimum pH of the enzyme is 8.0 - 8.5.

The substrate specificity of rat kidney glutathione synthetase has been examined in terms of the acceptability of  $\gamma$ -Glu-Cys and Gly derivatives (6). Since the amino acid moiety of  $\gamma$ -glutamyl-amino acids was limited to those sterically similar to cysteine (e.g.,  $\alpha$ -aminobutyrate, serine, alanine), the rat kidney enzyme is highly specific toward the cysteine moiety of  $\gamma$ -Glu-Cys. The D- $\gamma$ -glutamyl derivatives and N-methyl,  $\alpha$ -methyl,  $\beta$ -methyl, and  $\gamma$ -methyl-L- $\alpha$ -glutamyl derivatives of L- $\gamma$ -glutamyl-L- $\alpha$ -aminobutyrate were significantly active substrates. Moreover, N-acetyl-L-cysteine is also active. The L- $\gamma$ -glutamyl moiety of L- $\gamma$ -glutamyl-L- $\alpha$ -aminobutyrate can be modified considerably with retention of substrate activity. Three possible monomethyl derivatives of Gly ( L-Ala, D-Ala, and sarcosine ) are not active, nor is glycynamide. However, substantial activity is found with aminooxyacetate ( $\text{NH}_2\text{OCH}_2\text{COOH}$ ) and N-hydroxyglycine. Moore and Meister indicated that the substrate specificity of the *E. coli* B enzyme was probably similar to that of the rat kidney enzyme (8).

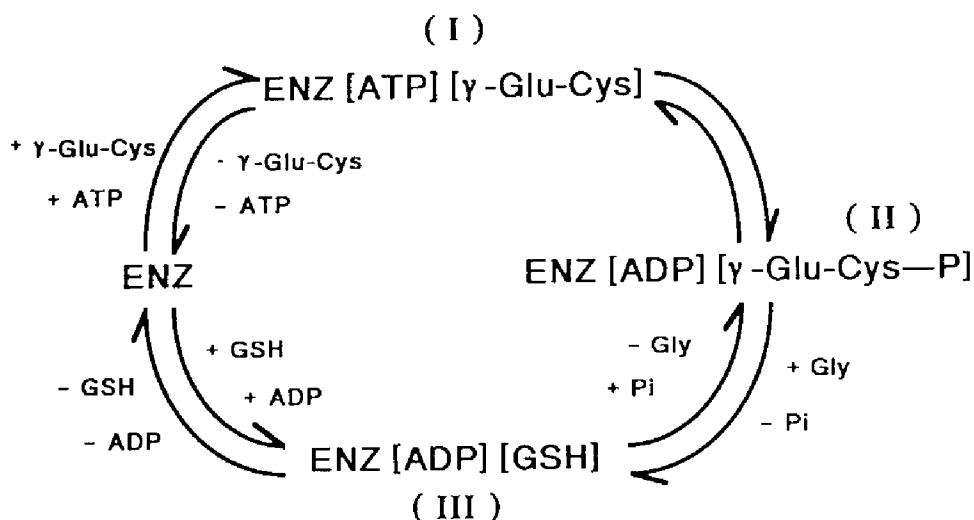


FIG. 0-1. Scheme of the reaction catalyzed by glutathione synthetase.

Reaction catalyzed by glutathione synthetase is supposed to proceed via enzyme-bound acylphosphate as an intermediate like those of glutamine synthetase and  $\gamma$ -glutamylcysteine synthetase (1). As the evidence for the formation such a acylphosphate,  $\gamma$ -glutamyl- $\alpha$ -aminobutyryl phosphate has been first obtained by using pulse-labeling technique (9,10) in the synthesis of  $\gamma$ -glutamyl- $\alpha$ -aminobutyrylglycine (ophthalmic acid) with glutathione synthetase. According to this model, the reaction proceeds in a following scheme (Fig. 0-1). The enzyme combines ATP and  $\gamma$ -Glu-Cys in the presence of  $Mg^{++}$  to yield a complex (I); phosphorylation of the  $\gamma$ -Glu-Cys yields complex (II), which consists of enzyme, ADP,  $Mg^{++}$ , and the  $\gamma$ -glutamylcysteinylphosphate. Subsequently, complex (II) can react with glycine to yield complex (III) and orthophosphate, from which GSH and ADP dissociate. In contrast, Wendel and Flohe

proposed another model by kinetic studies on purified bovine erythrocyte glutathione synthetase (11). Glutathione synthetase is first activated by ATP and  $Mg^{++}$ , then reacts with  $\gamma$ -Glu-Cys and Gly, randomly, to form the enzyme-three substrates complex (they called central complex), from which products, GSH, ADP and orthophosphate are released consecutively. In the latter model, complex (I), (II), and (III) in Fig. 0-1 are not distinguishable, thus the Gly binding and orthophosphate release are not ordered. Nevertheless, their kinetic data is not enough to exclude the scheme in Fig. 0-1.

In contrast to the information about the overall aspects of the catalytic pathway of glutathione synthetase reaction, the following informations remain unclear such as the binding property of substrates, particularly interactions of amino acid residues of the enzyme with substrates, and protein structures of the enzyme.

Involvement of cysteine residues in the catalytic function of glutathione synthetase has been suggested from chemical modification using sulfhydryl reagents. In the rat kidney enzyme, p-chloromercuribenzoate (pCMB) showed a marked inhibition, but N-ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoate) had no effect on catalytic activity (6). In the *E. coli* B enzyme, pCMB was also a potent inhibitor (7). Although, many details of the role of cysteine residues remain to be learned.

Therefore, precise knowledge for the reaction mechanism, in particular for the structure-function relationship is not yet available.

Recently, enzymology has been evolved and transformed by X-ray crystallography, genetic engineering, and computer technology (12). Protein engineering (13) is realized by those technology. However, the technology to do is already available but lack of principles of the protein design is a serious constraint in protein engineering.

In the present study, this author applied protein engineering techniques to examine the structure and function of glutathione synthetase from *E. coli* B, and come up with the principle of molecular design of protein. Primary structure of glutathione synthetase has been determined only for the enzyme from *E. coli* B by N-terminal amino acid sequence

analysis and by DNA sequence analysis of the *gsh* II gene (14). The *E. coli* B enzyme is a tetramer of identical subunit of 35,600 dalton (7). Each subunit of the *E. coli* B enzyme is composed of 316 amino acids including four cysteines. In Chapter I, Property of the ATP binding site was investigated by use of amino acid sequence homology (similarity). This approach is related to find out a module or motif structures participated in substrate binding. In Chapter II, the glutathione synthetase gene, *gsh* II was subcloned into overexpression plasmid, thus overproduction and rapid purification of the enzyme are achieved. In Chapter III, role of cysteine residues was analyzed by using chemical modification and site-directed mutagenesis. In Chapter IV, the *E. coli* B glutathione synthetase was crystallized and preliminary X-ray crystallographic studies were investigated. In addition, small angle X-ray scattering was measured about solution of the enzyme to compare in the aqueous solution state.

## CHAPTER I

### Similarity of *Escherichia coli* B Glutathione Synthetase with Dihydrofolate Reductase in Amino Acid Sequence and Substrate Binding Site.

#### I-1 INTRODUCTION

In order to find which amino acids of glutathione synthetase construct a substrate binding site and contribute to its catalytic reaction, the author compared the amino acid sequence of the *Escherichia coli* B glutathione synthetase with those of other enzymes. The amino acid sequence of glutathione synthetase is less similar with glutamine synthetase, glutathione reductase and ATPase. Amino acid sequence similarity search against a protein sequence database, however, revealed that the amino acid sequence of the *E. coli* B glutathione synthetase from Arg-55 to Ile-96 were highly similar to that of mouse dihydrofolate reductase from Gln-47 to Leu-89. In addition, the amino acid sequence of glutathione synthetase was locally similar to those of other bacterial and mammalian dihydrofolate reductases with a span of 40 residues (Fig. I-1), although glutathione synthetase and dihydrofolate reductase are quite different enzymes not only in their catalytic mechanisms, amide synthesis and hydrogenation, but also in their substrate requirements,  $\gamma$ -Glu-Cys, Gly and 7,8-dihydrofolate, respectively.

On the basis of these findings, the author tried to identify a functional role of the similar portion of the amino acid sequence in the *E. coli* B glutathione synthetase. First, the author improved a procedure of the purification of the *E. coli* B glutathione synthetase and obtained the enzyme preparation with a higher specific activity than those reported in other papers. With this enzyme preparation, the author found that the

FIG. 1-1. Amino acid alignment of the *E. coli* B glutathione synthetase with dihydrofolate reductase from ten origins with a span of 40 residues. Score is the measure of similarity based on the amino acid mutation data (Ref. 26). The values shown are the minimum value of each pair of sequences.

*E. coli* B glutathione synthetase was potentially inhibited not only by specific inhibitors of dihydrofolate reductase such as methotrexate (MTX) and trimethoprim, but also by its substrate 7,8-dihydrofolate. In particular, the inhibition by MTX was examined in detail, whereby it was proved that glutathione synthetase has an ATP binding site similar to a substrate binding site of dihydrofolate reductase. Then, the author analyzed 1) the folding of the similar portion of the polypeptide chain in the crystal structure of *Lactobacillus casei* dihydrofolate reductase and 2) the boundary of the similarity in the amino acid sequence with respect to the intron-exon boundary in mammalian dihydrofolate reductase genes. These analyses revealed that the similar portion of the polypeptide chain folds to make a structurally and genetically distinct subdomain; that is, this subdomain functions as a NADPH binding site in dihydrofolate reductase and as an ATP binding site in the *E. coli* B glutathione synthetase. Therefore, this study gives an experimental evidence that local similarity in amino acid sequences conserves a functional similarity even in enzymes having different catalytic mechanisms.

## I-2 EXPERIMENTAL PROCEDURES

**Chemicals** ATP, NADPH, and yeast glutathione reductase were purchased from Oriental Yeast (Osaka). 7,8-Dihydrofolate, methotrexate, and trimethoprim were from Sigma (St. Louis).  $\gamma$ -Glu-Cys was supplied by Kojin. DEAE-cellulose, Butyl-toyopearl 650M, Hydroxyapatite, Cellulofine GCL-2000 sf were supplied by Brown (Berlin), Toyo soda, Wako Chemical (Osaka), Chisso (Minamata), respectively. All other chemicals were the purest grades commercially available.

**Bacterial Strain and Cultivation** The gene for glutathione synthetase of *E. coli* B has been cloned onto vector plasmid pBR325 (15). *E. coli* C600 cells transformed by a recombinant plasmid pBR325 were grown on a nutrient medium (16) containing 0.005% Ampicillin at 30 °C for 20 h with reciprocal shaking.

**Determination of Enzyme Activity** The activity of glutathione synthetase was determined at 37 °C and pH 7.5. The enzyme solution, 5  $\mu$ l, was added to an assay medium composed of 5 mM  $\gamma$ -Glu-Cys, 10 mM Gly, 10 mM ATP, 10 mM MgSO<sub>4</sub>, and 100 mM Tris-HCl buffer (pH 7.5) in a total volume of 0.1 ml. To terminate the reaction, a test tube containing the reaction mixture was immersed in boiling water for 2 min. Glutathione was determined as the amount of 2-nitro-5-thiobenzoic acid which was formed by thiolysis of 5,5'-dithiobis-(2-nitrobenzoic acid) with glutathione in the presence of NADPH and glutathione reductase (17). One unit of enzyme was defined as the amount that catalyzed the formation of 1  $\mu$ mol of glutathione per min at 37 °C and pH 7.5.

Dihydrofolate reductase-like activity of glutathione synthetase was determined spectrophotometrically at 25 °C as the decrease in absorption of NADPH at 340nm (18). The assay system contained 77  $\mu$ M 7,8-dihydrofolate, 77  $\mu$ M NADPH, 7.7 mM 2-mercaptoethanol, and 39 mM potassium phosphate buffer (pH 7.5) in a total volume of 1.3 ml. Enzyme solution 50  $\mu$ l was added to initiate the reaction.



**Protein determination** Protein was determined by the method of Lowry (19). The concentration of glutathione synthetase in the purified preparation was calculated from the absorption coefficient  $A_{\text{cm}}^{1\%} = 9.02 \pm 0.44$  that was experimentally determined at 280 nm in 0.05 M potassium phosphate buffer pH 7.0 by the dry weight method (20).

**Purification of Glutathione Synthetase** All procedures were carried out at 0 - 4 °C, unless otherwise stated.

*Step 1. Preparation of crude extract.* The cells (156 g in wet weight) from 15 liters of culture were suspended in 500 ml of 10 mM Tris-HCl buffer (pH 7.2), and disrupted for 1 min  $\times$  3 with Dyno-Mill at 0 °C. Cell debris was removed by centrifugation at  $10,000 \times g$  for 15 min.

*Step 2. DEAE-cellulose column chromatography* Crude extract (660 ml, 6.9 g protein) was applied to a DEAE-cellulose column (6  $\times$  51 cm) equilibrated with 10 mM tris-HCl buffer (pH 7.2) and eluted from the column with a linear gradient established between the tris-HCl buffer and the same buffer containing 1.0 M KCl. Enzyme activity was eluted at a KCl concentration of approximately 0.2 M. The active fractions, 910 ml, were collected and added to 430 g of solid  $(\text{NH}_4)_2\text{SO}_4$  (70% saturation). After stirring for 1 h, the precipitate was collected by centrifugation at  $10,000 \times g$  for 20 min, dissolved in 100 ml of 50 mM potassium phosphate buffer (pH 7.0), then dialyzed against 5 liters of the buffer for 15 h.

*Step 3. Butyl-Toyopearl 650M column chromatography* The enzyme solution (122 ml) from step 2 was added to 21.5 g of solid  $(\text{NH}_4)_2\text{SO}_4$  (30 % saturation) and stirred for 1 h. The precipitate was removed by centrifugation at  $10,000 \times g$  for 15 min, and supernatant (150 ml) was applied to a Butyl-Toyopearl 650M column (5  $\times$  35 cm) pre-equilibrated with 50 mM potassium phosphate buffer containing 1.33 M  $(\text{NH}_4)_2\text{SO}_4$  (pH 7.0). The column was washed with 2 liters of the same buffer. Elution was carried out with linear concentration gradient of  $(\text{NH}_4)_2\text{SO}_4$  decreasing from 1.33 to 0 M. The active fractions, 280 ml,

were eluted at a  $(\text{NH}_4)_2\text{SO}_4$  concentration of approximately 0.83 M and concentrated to about 5 ml on a Amicon UM-10 membrane.

**Step 4. Cellulofine GCL-2000 column chromatography** The enzyme solution from step 3 was applied to a Cellulofine GCL-2000 sf column ( $3 \times 127$  cm) pre-equilibrated with 50 mM potassium phosphate buffer containing 0.1 M KCl and 5 mM  $\text{MgCl}_2$ . Elution was carried out with the same buffer. The active fractions, 18.4 ml, were pooled and dialyzed against 5 liters of 2.5 mM potassium phosphate buffer (pH 6.8).

**Step 5. Hydroxyapatite column chromatography** The dialyzate (25 ml) was applied to a hydroxyapatite column ( $2 \times 30$  cm) equilibrated with 2.5 mM potassium phosphate buffer (pH 6.8). Elution was carried out with a linear concentration gradient of potassium phosphate buffer from 2.5 to 100 mM. Active fractions, 59 ml, were eluted at approximately 60 mM potassium phosphate and concentrated to 2 ml on an Amicon UM-10 membrane. The enzyme preparations thus obtained was stored at  $-20^\circ\text{C}$  and used as the source of glutathione synthetase throughout this study.

**Electrophoresis** SDS-polyacrylamide gel electrophoresis was performed on 10 % acrylamide by the method of Laemmli (21). Proteins were detected by staining with Coomassie brilliant blue R-250.

**Reaction of Glutathione Synthetase with Inhibitors** The enzyme solution was pre-incubated with an inhibitor at  $37^\circ\text{C}$  for 10 min, then it was added into the assay mixture for glutathione synthetase. The mixture contains the inhibitor at the same concentration to the pre-incubation reaction.

**Kinetic Data Analysis** Apparent  $K_m$  and  $V_{max}$  values were determined by the non-linear least squares fitting method (22). The values for ATP were determined by the double-reciprocal plot because substrate inhibition by ATP appeared at higher concentrations.

**Similarity Search in Amino Acid Sequences** Local similarity in the amino acid sequence of the *E. coli* B glutathione synthetase was searched against a database, DDBJ, compiled by T. Ooi and H. Nakashima (Institute for Chemical Research, Kyoto University) (23). This database contains 3050 amino acid sequences of various types of proteins. Our search was limited to 1186 sequences relating to 280 different kinds of enzymes. A procedure which finds within longer sequences all subsequences that locally resemble one another was based on an algorithm developed by Goad and Kanehisa (24, 25). The measure of similarity is based on an amino acid mutation data (PAM matrix) among related proteins (26). Weight for a deletion was set equal to 8.

### I-3 RESULTS

**Purification of Glutathione Synthetase** Table I-I summarizes purification of the *E. coli* B glutathione synthetase from 15-liter culture

TABLE I-I  
Purification of Glutathione Synthetase : Summary.

	Total protein (mg)	Specific activity (unit <sup>a</sup> /mg)	Total activity (unit <sup>a</sup> )	Recovery (%)	Purity (-fold)
Crude extract	6,860	0.385	2,640	100	1
DEAE-cellulose	1,640	2.51	4,120	156	7
Butyl-Toyopearl	168	12.3	2,070	78.4	32
Cellulofine GCL-2000 sf	50.9	27.3	1,390	52.7	71
Hydroxyapatite	14.8	42.8	633	24.0	111

<sup>a</sup> One unit of enzyme was defined as the amount producing 1  $\mu$ mole of GSH per min under standard assay condition.

of *E. coli* C600. Overall purification achieved was approximately 110-fold with 24 % yield of the crude extract in the activity. Purified preparations gave a single band on the SDS-polyacrylamide gel electrophoresis and exhibited a constant specific activity, 42.8 unit/mg protein, which is a three- to six-fold activity of those reported previously (5, 6, 7).

Purification of the *E. coli* B glutathione synthetase took eight steps in the previous report (7). Butyl-Toyopearl 650M column chromatography in the present study reduced the steps required for purification and improved both the specific activity and recovery of the enzyme. Enzyme preparations from rat kidney have been effectively purified by affinity chromatography on Agarose-aminohexyl-N<sup>6</sup>-ATP (2, 6). But the *E. coli* B enzyme failed to be adsorbed on Agarose-aminohexyl-N<sup>6</sup>-ATP. This is due to the weak binding of ATP to the *E. coli* B enzyme when compared with the rat kidney enzyme. The apparent  $K_m$  values of ATP were 1.85 mM for the *E. coli* B enzyme (see Table I-III), but 0.03 mM for the rat kidney enzyme (6).

No activity as dihydrofolate reductase was observed with the *E. coli* B enzyme preparation.

***Inhibition of Glutathione Synthetase by Ligands of Dihydrofolate Reductase*** The purified preparation of the *E. coli* B glutathione synthetase was effectively inhibited by 7,8-dihydrofolate, MTX, and trimethoprim (Table I-II). While 7,8-dihydrofolate is a substrate of dihydrofolate reductase, MTX and trimethoprim are its inhibitors.

Stone and Morrison (27) recently reported that trimethoprim was different from MTX in the inhibition potency to bacterial and avian dihydrofolate reductases: MTX inhibits chicken liver as well as *E. coli* dihydrofolate reductases, inhibition constant  $K_i = 1.3$  and 3.6 nM, respectively. Trimethoprim, however, inhibits the chicken liver dihydrofolate reductase less than the *E. coli* dihydrofolate reductase by a 5000-fold,  $K_i = 3530$  and 0.49 nM, respectively. In this experiment, the *E. coli* B glutathione synthetase was inhibited more potent by MTX than by trimethoprim. Such difference in their inhibiting activities between MTX and trimethoprim could be ascribed to the fact that the amino acid sequence of the *E. coli* B glutathione synthetase is more homologous to

TABLE I-II

Effect of Ligands for Dihydrofolate Reductase on Glutathione Synthetase Activity

Glutathione synthetase was pre-incubated with the respective concentration of each ligand for dihydrofolate reductase in 100 mM Tris-HCl buffer (pH 7.5) at 37°C for 10 min, then the enzyme solution was added in to the assay mixture for glutathione synthetase, 5 mM  $\gamma$ -Glu-Cys, 15 mM Gly, 10 mM ATP, 10 mM  $\text{MgSO}_4$ , and 100 mM Tris-HCl buffer (pH 7.5) containing the ligand at the same concentration as in the pre-incubation reaction.

Ligands	Conc (mM)	Relative activity (%)
Control		100
MTX	0.01	75.0 (8.59) <sup>a</sup>
	0.1	36.0 (7.83)
Dihydrofolate	0.1	53.9 (3.82)
Trimethoprim	0.1	82.9 (2.22)
	0.3	58.2 (0.28)
NADPH	2.0	98.0 (0.85)

<sup>a</sup> Values in parentheses are standard error.

the mammalian dihydrofolate reductases than to the bacterial dihydrofolate reductases.

Cofactor NADPH, however, showed little inhibitory activity to glutathione synthetase.

***Mechanism of Inhibition of Glutathione Synthetase by Methotrexate*** Inhibition of the *E. coli* B glutathione synthetase by MTX was analyzed according to the method developed by Cleland (28, 29). Concentrations of ATP and MTX were varied under the fixed concentration of  $\gamma$ -Glu-Cys and Gly at 15 mM.

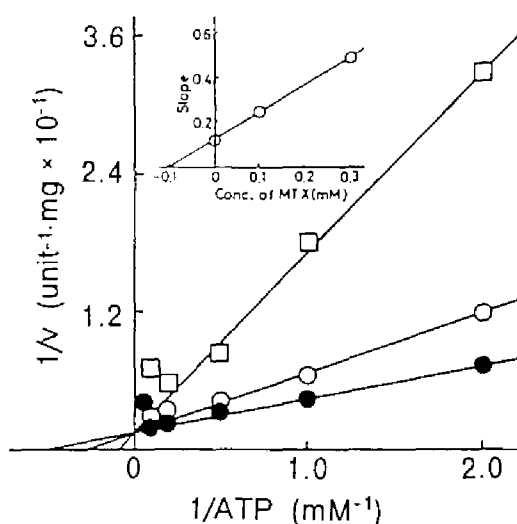


FIG. I-2. Double-reciprocal plots for MTX inhibition with respect to ATP. The assay mixture contained 15 mM  $\gamma$ -Glu-Cys, 15 mM Gly, 10 mM  $\text{MgSO}_4$ , 100 mM Tris-HCl buffer (pH 7.5), and varied concentration of ATP. MTX concentrations are 0 ( $\bullet$ ), 0.1 mM ( $\circ$ ), and 0.3 mM ( $\square$ ). Insert: Secondary plot for slope of double-reciprocal plots versus MTX concentration.

Although substrate inhibition by ATP appeared at the concentrations of ATP higher than about 15 mM, double-reciprocal plot  $1/v$  vs  $1/[\text{ATP}]$  gave a family of lines that converged on the  $1/v$  axis (Fig. I-2). This plot shows that the inhibition of glutathione synthetase by MTX is competitive with respect to ATP. When concentrations of  $\gamma$ -Glu-Cys and MTX were varied under the fixed ATP and Gly concentration, lines in double-reciprocal plot  $1/v$  vs  $1/[\gamma\text{-Glu-Cys}]$  converged on the  $1/[\gamma\text{-Glu-Cys}]$  axis (Fig. I-3). From this plot, it is shown that inhibition by MTX is noncompetitive with respect to  $\gamma$ -Glu-Cys. When glutathione synthetase was inhibited by MTX in the presence of Gly as the varied substrate, parallel lines were obtained in double-reciprocal plot  $1/v$  vs  $1/[\text{Gly}]$  (Fig. I-4). Therefore, inhibition by MTX is uncompetitive with respect to Gly. The values of inhibition constant  $K_i$  of MTX with respect to ATP,  $\gamma$ -Glu-Cys, and Gly are summarized in Table III with their apparent  $K_m$  values. The  $K_i$  values of MTX are almost constant, 0.1 mM, independent of a substrate varied and are 20 - 50 times smaller than  $K_m$  values of ATP,  $\gamma$ -Glu-Cys, and Gly.

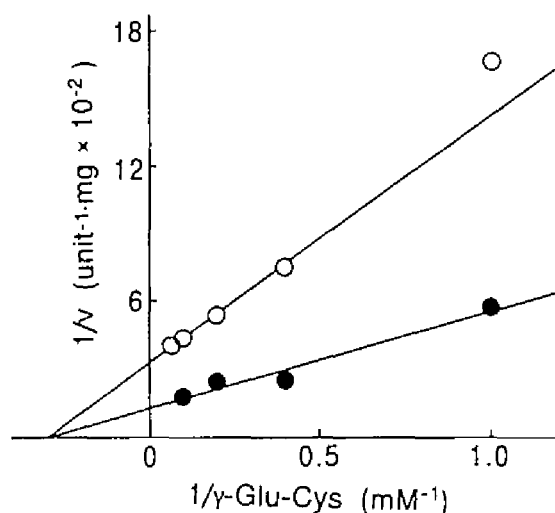


FIG. I-3. Double-reciprocal plots for MTX inhibition with respect to  $\gamma$ -Glu-Cys. The assay mixture contained 10 mM ATP, 15 mM Gly, 10 mM  $\text{MgSO}_4$ , 100 mM Tris-HCl buffer (pH 7.5), and varied concentration of  $\gamma$ -Glu-Cys. MTX concentrations are 0 (●) and 0.1 mM (○).

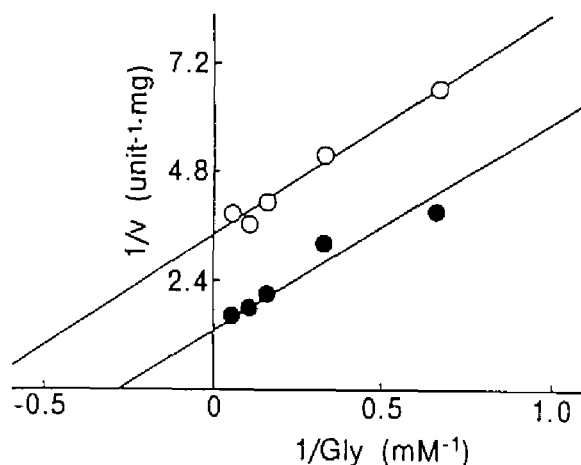


FIG. I-4. Double-reciprocal plots for MTX inhibition with respect to Gly. The assay mixture contained 10 mM ATP, 15 mM  $\gamma$ -Glu-Cys, 10 mM  $\text{MgSO}_4$ , 100 mM tris-HCl buffer (pH 7.5), and varied concentrations of Gly. MTX concentrations are 0 (●) and 0.1 mM (○).

TABLE I-III.

The Values of Inhibition Constant  $K_i$  and Inhibition Type of MTX with respect to ATP,  $\gamma$ -Glu-Cys, and Gly with Their Apparent  $K_m$  Values.

	$K_m$ (mM)	$K_i$ (mM)	Type <sup>a</sup>
ATP	1.85(0.39) <sup>b</sup>	0.10(0.01)	Competitive
$\gamma$ -Glu-Cys	3.33(0.37)	0.07(0.04)	Noncompetitive
Gly	3.53(0.70)	0.10(0.02)	Uncompetitive

<sup>a</sup>Inhibition types were determined from Fig. I-2, I-3, and I-4.

<sup>b</sup>Values in parentheses are standard error.

## I-4 DISCUSSION

Meister analyzed reactions of glutathione synthetase and proposed that the reaction proceeded by the ordered mechanism containing three states of the enzyme, E, F, and F' (Fig. I-5) (1). Glutathione synthetase in a substrate free condition, E, combines with ATP and  $\gamma$ -Glu-Cys in the presence of  $Mg^{2+}$  to yield a ternary complex. This complex proceeds into the state F as the bound  $\gamma$ -Glu-Cys is phosphorylated by ATP.

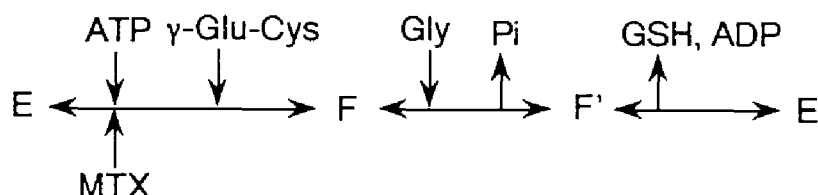


FIG. I-5. The proposed reaction mechanism of glutathione synthetase (Ref. 1) and MTX inhibition. Glutathione synthetase in a substrate free state (E), a ternary complex with ATP and  $\gamma$ -Glu-Cys (E'), a complex with phosphorylated  $\gamma$ -Glu-Cys (F), and a state in which glutathione is formed (F').



The resulting phosphorylated  $\gamma$ -Glu-Cys reacts with Gly to form a complex in state F', from which ADP,  $P_i$ , and glutathione dissociate. Then, the enzyme returns to the initial state E. The types of inhibition by MTX summarized in Table I-III is consistent with this reaction scheme only when MTX binds to an ATP binding site of the *E. coli* B enzyme. In addition, inhibition by 7,8-dihydrofolate suggests that the ATP binding site is similar to a substrate binding site of dihydrofolate reductase.

Here arises a question of whether the amino acid sequence similarity found in this study was responsible for the binding of MTX to an ATP binding site of the *E. coli* B glutathione synthetase. This question is considered in the following discussion by analyzing crystal and gene structures of dihydrofolate reductases in relation to the similar portion of their amino acid sequences, because these structures of glutathione synthetase are unknown. Crystal structure of dihydrofolate reductase complexed with its ligands has been analyzed for the enzymes from *L. casei*, *E. coli* (30, 31), and chicken liver (32). Although amino acid sequences of dihydrofolate reductase are only 25 % identical between animal and bacterial enzymes, overall backbone chain folding of chicken liver dihydrofolate reductase is very similar to that observed in bacterial enzymes. About 70 % of the additional residues present in the avian enzyme occur in three loops that were placed far from its substrate and cofactor binding sites.

First, the interactions of the similar portion of the polypeptide chain of dihydrofolate reductase with MTX and NADPH are estimated by counting the number of amino acid residues involved in the binding of the ligands. The similar portion interacts with NADPH at the adenosine-2',5'-diphosphate moiety and with MTX at the p-aminobenzamide moiety. Filman and Bolin et al (30, 31) have extensively studied the crystal structure of the ternary complex of the *L. casei* dihydrofolate reductase with NADPH and MTX. They listed the all amino acid residues that were contact with the bound NADPH and ATP molecules. According to their listing, eleven amino acid residues locate in contact with the adenosine diphosphate moiety of the bound NADPH molecule by van der Waals and hydrogen bonding interactions (30). Eight residues among them belong

to the similar portion (from Ile-38 to Val-75) of the polypeptide chain. Further more, among five amino acid residues which are involved in the binding of the p-aminobenzamide moiety of MTX (31), only three residues are present in the similar portion. Therefore, it is safely concluded that the similar portion of the polypeptide chain in the *L. casei* dihydrofolate reductase interact with the binding of the adenosine diphosphate moiety of NADPH rather than with MTX.

Secondary, the author analyzes the tertiary structure of the similar portion of the polypeptide chain of dihydrofolate reductase in terms of the correlation between the protein and gene structures of the enzyme. According to a molecular evolution theory of enzymes, functional domains in enzymes are related to the intron/exon structure of the enzyme genes (33, 34). DNA sequences of mammalian genes of dihydrofolate reductase have been reported for mouse and human genes (35, 36, 37). The coding portions of the two genes are separated into six exons with identical exon boundaries at amino acid residues 30, 46, 81, 123, and 162. The similar portion of the amino acid sequence of the mammalian dihydrofolate reductases was from Gln-47 to Leu-89. This corresponds to the portion coded by exon 3 (from Gly-45 to Lys-80). Since the boundaries of sequence similarity is almost same to those of exon 3 in mammalian dihydrofolate reductases, the similar portion is a genetically distinct sequence.

The correlation between the protein and gene structures of an enzyme has been analyzed by examining whether polypeptide chain coded by an exon (or exons) folds to make a structural unit distinct from other parts of the structure of the enzyme. For example, each exon in hemoglobin and lysozyme has been correlated to a protein structure called 'module' (38, 39). This is visualized on a distance map, which is a plot of the distance between the *i*-th and *j*-th  $\alpha$ -carbon atoms in a triangular space (40). Protein folding of the *L. casei* dihydrofolate reductase is shown on a  $C\alpha$ - $C\alpha$  distance map, along which six exons of the mouse gene are placed by aligning the amino acid sequence of the mouse enzyme to that of the *L. casei* enzyme (Fig. I-6).

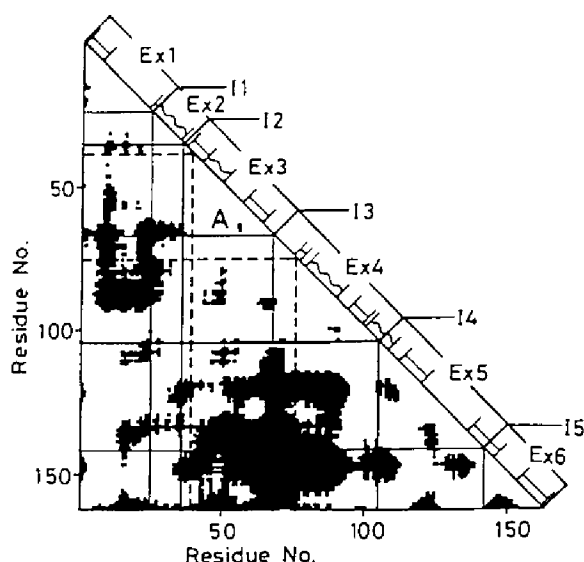


FIG. I-6. Distance map of a ternary complex of dihydrofolate reductase from *L. casei* with MTX and NADPH. The distances between the *i*-th and *j*-th  $\alpha$ -carbon atoms were calculated from the crystal structure data in Protein Data Bank (Brookhaven National Laboratory, USA). Dark regions represented the pairs of  $\alpha$ -carbon atoms separated by  $>25$  Å. Symbols (—) and (—) are  $\alpha$ -helix and  $\beta$ -sheet structures, respectively. Five introns (I1-I5) and six exons (Ex1-Ex6) are placed on the distance map by aligning the amino acid sequence of mammal dihydrofolate reductase with that of the *L. casei* enzyme. Dots are the boundary of similar sequence with glutathione synthetase. The area A is the folding of the similar sequence.

Dark regions represent the pairs of  $C^\alpha$  atoms separated by  $>25$  Å. Two large, dark regions in the distance map suggest that there are three structural segments divided from each other by introns 2 and 4. The segment constructed by the polypeptide chain coded by exons 3 and 4 is a distinct structural unit separated in space from the other two segments. In particular, distance between a pair of the  $C^\alpha$  atoms in the polypeptide chain from Ile-38 to Val-75 is less than 25 Å as shown in a triangular area A in Fig. I-6. This part of the polypeptide chain is folded compactly. As to secondary structures of the enzyme, two  $\beta$ -sheets and one  $\alpha$ -helix are included in the region coded by exon 3. The above analyses show that the polypeptide chain coded by exon 3 is best in the correlation between intron/exon and protein structures as well as in the sequence similarity with glutathione synthetase. From the above analyses, the similar portion

of the polypeptide chain in dihydrofolate reductase is defined as a distinct functional and structural unit that is responsible for the binding of NADPH.

Thirdly, the author discusses the functional similarity between the NADPH binding site of dihydrofolate reductase and the ATP binding site of the *E. coli* B glutathione synthetase from the view of the chemical structures of ligands. Chemical structures of NADPH and ATP have a common substructure, adenosine phosphate moiety. The binding site of this substructure on the two enzymes might have similar affinity and specificity to other ligands. This is shown by the following observations. Stone and Morrison (41) have reported that 7,8-dihydrofolate caused substrate inhibition by binding to the NADPH binding site of dihydrofolate reductase. 7,8-Dihydrofolate could bind to this site because it has 2-aminopteridine moiety that is similar to adenosine moiety of NADPH. By the same way, 7,8-dihydrofolate and MTX are similar to ATP in their substructures, so they showed strong affinity to the ATP binding site of the *E. coli* B glutathione synthetase. NADPH molecule is too large in the size to fit the ATP binding site of the *E. coli* B enzyme (Table I-II). Therefore, the ATP binding site of the *E. coli* B enzyme is functionally resemble to the binding site of the adenosine diphosphate moiety of NADPH on dihydrofolate reductase, and must be constructed by the similar portion of the polypeptide chain.

In conclusion, the author experimentally related the sequence similarity found between two different types of enzymes to the similarity in their cofactor binding sites. The polypeptide chain from Arg-55 to Ile-96 of the *E. coli* B glutathione synthetase, which is similar to dihydrofolate reductase, must be a genetically and structurally distinct subdomain responsible for the binding of ATP. The purpose of amino acid sequence similarity search is usually to get the best alignment of two sequences of proteins which have same physiological function but different in their origins. Similarity search between proteins with different biological functions, however, is a useful tool to discover their unknown functions and structural units.

## CHAPTER II

# Overexpression of the *Escherichia coli* B Glutathione Synthetase Gene, *gsh* II, and Rapid Purification of the Enzyme

### II-1 INTRODUCTION

A variety of strong promoters that are segment of DNA which direct RNA polymerase binding and initiation of transcription of a gene, have been developed and applied for construction of high-level expression plasmids in *E. coli* (42). *tac* Promoter is a fusion of the consensus -35 sequence from the *trp* promoter and the consensus -10 sequence from the *lac* UV5 promoter (43, 44). Overexpression of prokaryote genes have been attained to 20 - 30 % of the total cell protein by subcloning downstream of the *tac* promoter (44, 45, 46, 47).

In this Chapter, the author describes 1) construction of an overexpression plasmid for *gsh* II gene by use of pKK223-3 that contains a polylinker site flanked by the *tac* promoter and the *rmb* transcription terminators (48) ; 2) kinetics and extent of induction, and stability of the plasmid; and 3) purification of the glutathione synthetase from *E. coli* cells containing the plasmid.

### II-2 EXPERIMENTAL PROCEDURES

**Bacterial Strains, Phages, and Plasmids** Overexpression plasmid pKK223-3 containing the *tac* promoter (43) was obtained from Pharmacia (Uppsala). Plasmid pGS400 is a pBR325 derivative containing a 1.8 kilobase pairs *Bam* HI fragment of glutathione synthetase gene from

*E. coli* B,*gsh* II (15). A host for pKK223-3 plasmids and M13 phages was *E. coli* JM109 (*rec A* 1 *end A* 1, *gyrA* 96, *thi*, *hsdR* 17, *supE* 44, *relA* 1,  $\lambda^{-1}$ ,  $\Delta(lac-proAB)$ , [F', *traD* 36, *pro AB*, *lacI*<sup>q</sup>Z $\Delta$ M15]) (49).

**Chemicals** Restriction enzymes, T<sub>4</sub>DNA ligase, and Klenow fragment of DNA polymerase I were provided by TAKARA SHUZO CO.,LTD(Kyoto). ATP, NADPH and yeast glutathione reductase were purchased from Oriental Yeast Co.,(Tokyo).  $\gamma$ -L-glutamyl-L-cysteine( $\gamma$ -Glu-Cys) was supplied by Kojin Co.,(Tokyo). DEAE-Cellulofine A-800, Cellulofine GCL-1000 m were gifted from Chisso Co.,(Minamata). Isopropyl  $\beta$ ,D-thiogalactopyranoside(IPTG) was purchased from Nakalai Tesque Inc.(Kyoto). All other chemicals used were of the highest grade available.

**Construction of Overexpression Vectors** Phage M13mp8-*gsh*II, containing the wild-type entire *gsh* II gene, was cleaved at *Eco*O109 site located 13 bases 3'-distal from Pribnow box of the *gsh* II gene and treated with Klenow fragment to repair end of the terminal, then cleaved at *Pst*I site (Fig. II-1). The entire *gsh* II gene was isolated on 5% polyacrylamide gel electrophoresis and subcloned into *Sma* I and *Pst* I sites of pKK223-3. This derivative of pKK223-3 was designated pKGS00.

**Growth of the cells and preparation of cell-free extracts** *E. coli* cells were grown in the LB medium(1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 50  $\mu$ g/ml ampicilline, the pH was adjusted to 7.5, at 37 °C with reciprocal shaking. Culture were grown to nearly log phase ( $A_{600} = 0.7 \text{ cm}^{-1}$  about 2.2 hr ), induced with IPTG (1 mM, final concentration), and then grown for 19 hours. Cells (from 500 ml culture) were harvested by centrifugation at  $10,000 \times g$  for 10 min, washed with 50 ml of 20 mM Tris-HCl buffer, pH 7.3 containing 0.05 M NaCl and 5 mM MgCl<sub>2</sub>, resuspended in 20 ml of the above buffer, and sonicated. The suspension was centrifuged at  $15,000 \times g$  for 20 min to remove cellular debris.

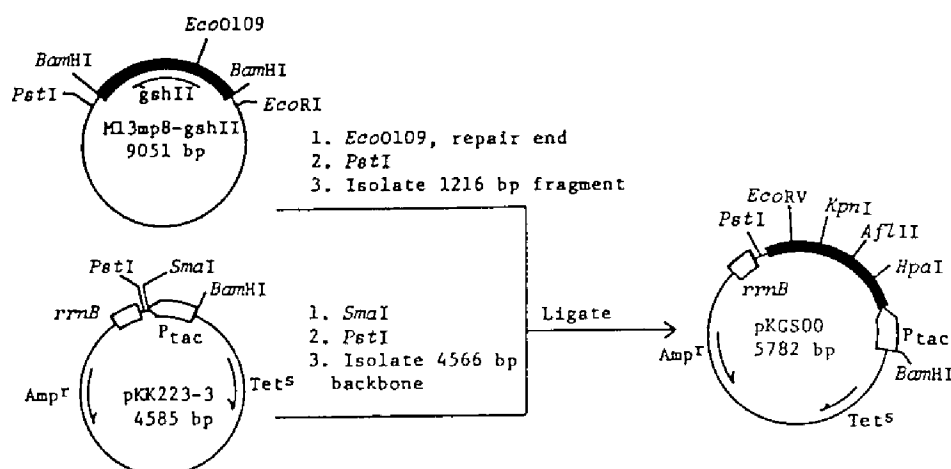


FIG. II-1. Construction of the overexpression vector of *gsh II* gene. Open bar arrowhead represents the *tac* promoter and indicates the direction of transcription. The open bar represents the *rrnB* terminators. The solid bar represents the subcloned fragment of the *gsh II* gene.

**Purification of the glutathione synthetase** All procedures were carried out at 0 - 4 °C.

**Step 1: DEAE-Cellulofine column chromatography** Cell-free extract (20 ml, 380 mg protein) was applied to a DEAE-Cellulofine A-800 column (2.4 × 48 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.2 containing 5 mM MgCl<sub>2</sub> and 50 mM NaCl. After washing the column with 500 ml of the same buffer, elution was carried out with a linear gradient of 0.05 - 0.3 M NaCl (0.5 l each in the mixing chamber and in the reservoir). The active enzyme was eluted at a NaCl concentration of approximately 0.15 M, and concentrated to about 5 ml on a Amicon PM-10 membrane.

**Step 2: Cellulofine GCL-1000 chromatography** The enzyme solution from step 1 was applied to a Cellulofine GCL-1000 m column (2.4 × 96 cm) equilibrated with 50 mM potassium phosphate buffer, pH 6.8, containing 0.1 M NaCl and 5mM MgCl<sub>2</sub>. Elution was carried out with the same buffer. The active fraction was concentrated on the Amicon membrane and dialyzed against 50 mM potassium phosphate buffer, pH 6.8 containing 5 mM MgCl<sub>2</sub>. The enzyme preparation (1 - 2 % concentration of protein) thus obtained was stored at 4 °C.

**Enzyme activity assay** The enzyme solution, 5  $\mu$ l, was added to an assay medium composed of 5 mM  $\gamma$ -Glu-Cys, 15 mM Gly, 10 mM ATP, 10 mM  $\text{MgSO}_4$ , and 100 mM Tris-HCl buffer, pH 7.5, in a total volume of 0.1 ml(50). Glutathione was determined as the amount of 2-nitro-5-thiobenzoate anion which was formed by thiolysis of 5,5'-dithiobis(2-nitrobenzoate) (DTNB) with glutathione in the presence of NADPH and glutathione reductase (17). One unit of enzyme was defined as the amount that catalyzed the formation of 1  $\mu$ mole of glutathione per min at 37 °C.

**Protein assays** Protein concentration of cell-free extract was measured by the method of Lowry *et al.* (19) with crystalline bovine serum albumin as the standard. The concentration of the glutathione synthetase in the purified preparation was determined from  $A_{280}^{1\%} = 9.02 \text{ cm}^{-1}$  in 50 mM potassium phosphate buffer, pH 7.0 (50).

**Polyacrylamide gel electrophoresis** Crude extracts as well as purified preparations were analyzed by 10 % SDS polyacrylamide gel electrophoresis according to the method of Laemmli (21). To determine the relative amount of the glutathione synthetase protein, the gels were stained with Coomassie Brilliant Blue R-250 and scanned at 570 nm using a Model CS910 chromatoscanner (Shimadzu Co.).

**Amino-acid Analysis** Amino-acid analysis was performed by following the method of Spackman *et al.* (51) with Hitachi model 835 amino-acid analyzer. Sample of 2.8 n moles were hydrolyzed with 6 N HCl (constant boiling) *in vacuo* at 110 °C for 22 and 72 hours. Cysteine was determined as cysteinic acid after oxidation with performic acid (52).

## II-3 RESULTS

### *Characterization of the expression of the gsh II in pKGS00*

The Growth of *E. coli* JM109 transformed with pKGS00, a derivative of pKK223-3 containing the *gsh II* gene, and the induction of the expres-



sion of the *gsh II* were shown in Fig. II-2. In proportion to cell growth, glutathione synthetase specific activity increased to nearly 22.0 unit/mg in cell-free extract from 19 hr culture induced with IPTG. This activity was 660-fold of that observed in chromosomal levels of the glutathione synthetase specific activity (0.03 unit/mg) of *E. coli* JM109 in the absence of pKGS00 transformation. SDS-Polyacrylamide gel electrophoresis stained with Coomassie Brilliant Blue R-250 was used to measure the time course of glutathione synthetase protein synthesis in *E. coli* JM109 containing pKGS00 induced with IPTG. Relative amounts of the glutathione synthetase protein increased with specific activities and expression of *gsh II* on pKGS00 reached to a maximum of about 53 % of the total cellular protein based on the intensities of protein bands (Fig. II-3).

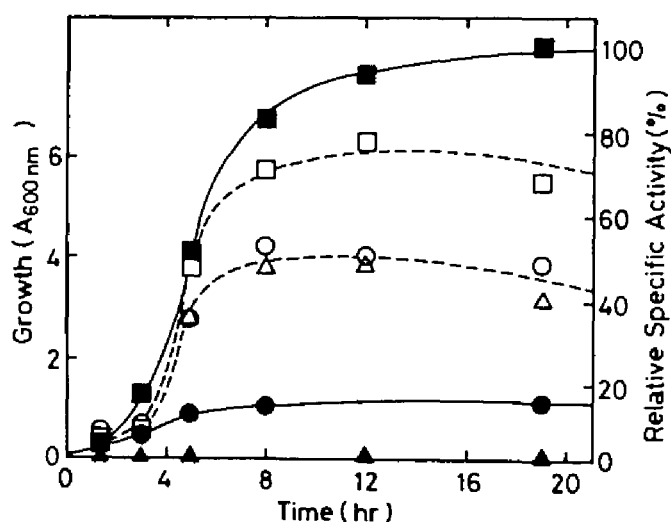


FIG. II-2 Growth of the *E. coli* JM109 transformed with pKGS00, and the induction of the expression of the *gsh II*. Cells were grown in 500 ml of LB medium, containing 50  $\mu$ g of ampicillin/ml, pH was adjusted to 7.5, at 37  $^{\circ}$ C with reciprocal shaking. After induction for 2.2 hour ( $A_{600} = 0.7 \text{ cm}^{-1}$ ) at 37  $^{\circ}$ C, IPTG (1 mM, final concentration) was added to one of the culture but not the other. At prescribed time intervals, 10 ml aliquots were withdrawn for preparation of cell-free extract and specific activities of glutathione synthetase were determined. After 19 h incubation with IPTG, The value of the specific activity was 22.0. This values has been assigned as 100, and the values for the other incubation times were related to that of it. For +IPTG :  $\square$ , growth;  $\blacksquare$ , activity. For -IPTG :  $\circ$ , growth;  $\bullet$ , activity. Growth of the *E. coli* JM109, not transformed was also examined and indicated by  $\triangle$ , growth;  $\blacktriangle$ , activity.

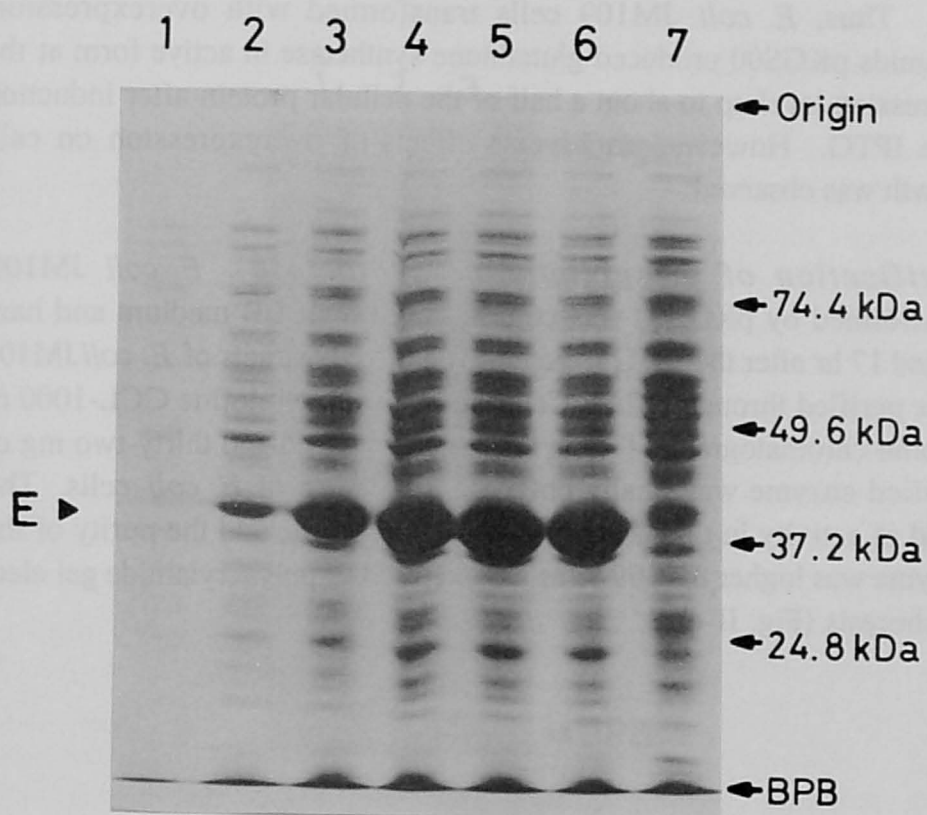


FIG. II-3 SDS-Polyacrylamide gel analysis of cell-free extract from induced culture. *E. coli* JM 109 transformed with pKGS00 were cultured and induced by IPTG as described in legend to Fig. II-2. Lanes 1 through 6 were grown for 1.5, 3, 5, 8, 12, 19 hours, respectively. Lane 7 was cell-free extract from *E. coli* JM 109 cells not transformed, were grown for 19 hours. E, glutathione synthetase; BPB, bromophenol blue.

The expression of the *gsh* II gene was also estimated from the comparison of specific activities between crude and purified preparations. Glutathione synthetase activities of the cell-free extract and purified preparation were 22.0 unit/mg and 42.8 unit/mg protein, respectively (Chapter I). Then, glutathione synthetase in the cell-free extract was estimated as about 51 % ( $=22.0 \div 42.8 \times 100$ ) in purity based on its specific activity.

Thus, *E. coli* JM109 cells transformed with overexpression plasmids pKGS00 produced glutathione synthetase in active form at the expression level up to about a half of the cellular protein after induction with IPTG. However, no adverse effects of overexpression on cell growth was observed.

**Purification of the glutathione synthetase** *E. coli* JM109 transformed by pKGS00 was cultured in 500 ml LB medium and harvested 17 hr after the IPTG induction. Cell-free extracts of *E. coli* JM109 were purified through DEAE-Cellulofine, then Cellulofine GCL-1000 m column chromatography (Table II-I). One hundred and thirty-two mg of purified enzyme was finally obtained from 2.7 g of *E. coli* cells. The yield of activity in this procedure was about 65 %, and the purity of the enzyme was higher than 99 % as judged by SDS-polyacrylamide gel electrophoresis (Fig. II-4).

TABLE II-I  
Purification of glutathione synthetase : Summary.

	Total protein (mg)	Specific activity (unit <sup>a</sup> /mg)	Total activity (unit <sup>a</sup> )	Recovery (%)	Purity (-fold)
Crude extract	380	22.0	8,360	100	1
DEAE-Cellulofine A-800	170	38.5	6,550	78.3	1.75
Cellulofine GCL-1000 m	132	41.3	5,450	65.2	1.88

<sup>a</sup> One unit of enzyme was defined as the amount producing 1  $\mu$  mole of GSH per min under standard assay condition.

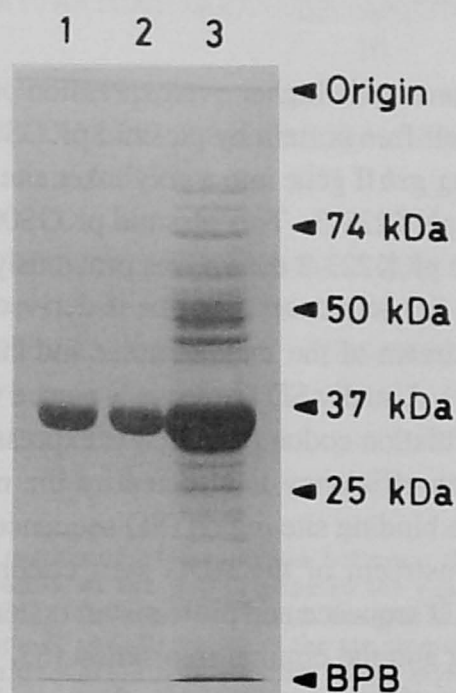


FIG. II-4 SDS-Polyacrylamide gel analysis of enzyme purification. Lane 1, purified preparation by Cellulofine GCL-1000 sf; lane 2, DEAE-Cellulofine A800 m fraction; lane 3, Cell-free extract.

**Amino-Acid Analysis of The Enzyme** Amino-acid composition of the purified preparation was as follows (figures in parentheses are values expected from the base sequence of the gene): Ala 21.9(22), Arg 19.2(19), Asp 31.3(32), Cys 4.0(4), Glu 39.0(38), Gly 25.3(25), His 4.2(4), Ile 24.9(26), Leu 32.2(33), Lys 18.4(18), Met 9.9(10), Phe 11.2(11), Pro 16.2(17), Tyr 9.0(9), Val 15.1(15). The agreement between these values and the values deduced from the nucleotide sequence was within 3 % of experimental error.

## II-4 DISCUSSION

The author attained unusually higher overexpression of the *gsh* II gene up to 50% of the total cell-free protein by plasmid pKGS00 which was constructed by subcloning *gsh* II gene into a polylinker site downstream of *tac* promoter in plasmid pKK223-3. This plasmid pKGS00 is different in SD sequences from those pKK223-3 derivatives previously reported. That is, pKGS00 carries two SD sequences; the one is derived from the  $\beta$ -galactosidase gene downstream of the *tac* promoter and the other is from the *gsh* II gene (Fig. II-5). Usually SD sequence is placed with an appropriate spacing before the initiation codon for efficient expression of a gene in *E. coli*. The translation efficiency is affected by the mRNAs secondary structure of ribosome binding site region (SD sequence) and the first several tens bases downstream of the AUG start codon, because single-strandedness of the SD sequence and protein start codon may be important for ribosome binding and the optimal translation (53, 54, 55, 56, 57, 58, 59). In certain cases of poor expressions, these region makes a base-paired stem and loop (hairpin) structure. The author analyzes several possible secondary structures of mRNA over 130 base length from 3'-terminal to the start codon of *gsh* II gene by SDC-GENETYX (Software Development Co.) genetic information management software. Fig. II-6 shows one of most probable secondary structure of mRNA at these regions. It is indicated that the second SD sequence derived from *gsh* II gene, rather the first one from  $\beta$ -galactosidase, is near the start codon and affect the translational efficiency. The second SD sequence (GAAG) is located neither in loop nor stem structure, but start codon AUG is positioned in a root of a little stem structure. However, this stem merely contains a double-stranded region of 4 base-pairs and three of these are less stable A and T pairs. Consequently, the ribosome binding region between the second SD sequence and start codon of *gsh* II gene may form a single-stranded state. Schottel et al. suggested that base pairing of the region located between the SD sequence and the AUG start codon, or of the start codon itself, did not substantially affect the efficiency of translation,

```

-----+-----+-----+-----+
TGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATT
      -35                      -10
-----+-----+-----+-----+
GTGAGCGGATAACAATTTACACAGGAAACAGAATTCCCG
                        SD1
-----+-----+-----+-----+
ACCTCGCGTTTTGCGTACAGAGACAACCTGCGCTCACCGCC
-----+-----+-----+-----+
ATTACCGCGCTACAAGTACGATTTGGCGATTTGGGCTAAC
-----+-----
GGAGAAGAATAATGATC
      SD2      Met Ile

```

FIG II-5. Nucleotide sequence of the region between the *tac* promoter and translational start codon of the *gsh* II gene in the plasmid, pKGS00. The fusion point of the pKK223-3 vector DNA with the *gsh* II gene was indicated by arrow. -35, and -10 were -35 and -10 regions of the *tac* promoter. SD1 and SD2 were the SD sequence from pKK223-3 and from the leader region of the *gsh* II gene respectively.

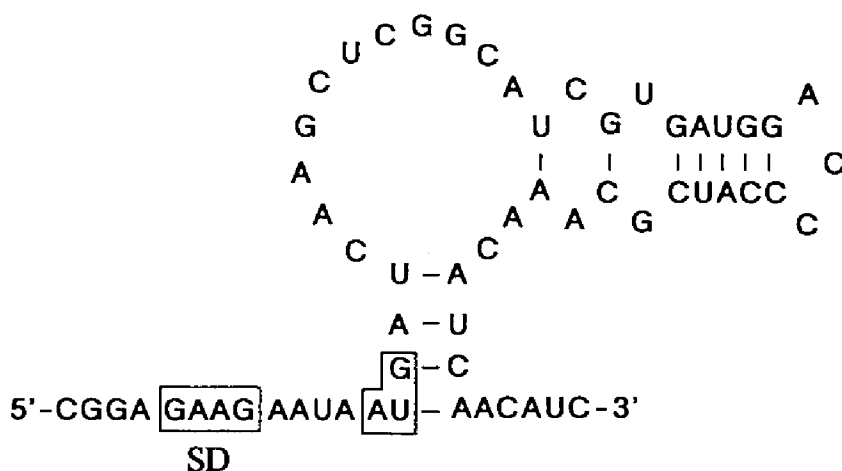


FIG II-6. A possible mRNA secondary structure model for the region around the SD sequence and the start codon of the *gsh* II gene in the pKGS00. Boxed areas indicated SD sequence and start codon AUG.

though the base pairing SD sequence with neighboring nucleotides reduces the translation efficiency (58). In addition, deBoer et al. suggested that the presence of the C and G residues following the SD sequence reduced the translation efficiency (60). The *gsh II* gene of pKGS00 has no C or G residues in the spacer between the SD sequence and the start codon (Fig. II-5, Fig. II-6). According to these reasons, pKGS00 exhibited the overexpression up to 50 % of the soluble cellular protein.

Purification of the glutathione synthetase from *E. coli* B previously took 4 steps and achieved with only 24 % yield of the crude extract in Chapter I. On the other, owing to this overproduction of the glutathione synthetase by pKGS00, a good yield (65%) has been attained by a simplified purification scheme (Table II-I) containing only 2 steps. This is necessary for the following experiments (Chapter III) examining the reactivity of mutant glutathione synthetases constructed by the site-directed mutagenesis.

## CHAPTER III

### Role of Cysteine Residues in Glutathione Synthetase from *Escherichia coli* B

#### Chemical Modification and Oligonucleotide Site-Directed Mutagenesis

#### III-1 INTRODUCTION

It is essential for understanding the mechanism of enzymatic catalysis to identify the amino acid residues which comprise the active site of an enzyme and assign their specific roles. *Escherichia coli* B glutathione synthetase is composed of four identical subunits; each subunit contains four cysteine residues (Cys-122, 195, 222, and 289). Involvement of cysteine residues in the catalytic activity of glutathione synthetase has been suggested from chemical modification using sulfhydryl reagents (1,4,6).

One of preliminary experiments showed that DTNB was an inhibitor of the *E. coli* B enzyme as potent as pCMB and that only one mole of DTNB reacted with each subunit to induce complete inactivation of this enzyme. This suggests that among four cysteine residues present in a subunit, only one sulfhydryl group is reactive with DTNB.

In this study, the author has attempted to understand the structural and functional roles of the four sulfhydryl groups of the *E. coli* B glutathione synthetase by giving answers to questions such as 1) which sulfhydryl group is reactive with DTNB, 2) whether other sulfhydryl reagents react with the same sulfhydryl group to DTNB reacts, and 3) whether these sulfhydryl groups are essential for catalytic activity.

To identify the sulfhydryl group(s) reactive with sulfhydryl reagents, the author had to prepare at least four different mutant enzymes in which only one of four cysteine residues was abolished. A set of mutant genes, in which a codon of cysteine was replaced with that of alanine, were



constructed from the wild-type *gsh II* gene of *E. coli* B by site-directed mutagenesis. Other three mutant genes containing only two or no cysteine codons were also constructed. Each mutant of the *gsh II* gene was subcloned into overexpression plasmid pKGS00 in which a mutant gene is controlled by the *tac* promoter (43) and inducible by IPTG.

### III-2 EXPERIMENTAL PROCEDURES

**Bacterial Strains, Phages, and Plasmids** The plasmid pKGS00 was a overexpression plasmid for the glutathione synthetase gene (*gsh II*) of *E. coli* B. Construction of the pKGS00 has been described (in Chapter II) were as follows :Phage M13mp8w-*gshII*, containing the wild-type entire *gsh II* gene, was cleaved at *Eco*O109 site located 13 bases 3'-distal from Pribnow box of the *gsh II* gene and treated with Klenow fragment to repair end of the terminal, then cleaved at *Pst*I site. The entire *gsh II* gene was isolated on 5% polyacrylamide gel electrophoresis and subcloned into *Sma*I and *Pst*I site of pKK223-3. Plasmid pGS400 is a pBR325 derivative containing a 1.8 kilobase pairs *Bam* HI fragment of glutathione synthetase from *E. coli* B, *gsh II* (15). M13mp8w is a M13mp8 derivative lacking of amber mutation in genes I and II. Bacterial strains for oligonucleotide site-directed mutagenesis were *E. coli* JM105 (*thi*, *rpsL*, *endA*, *sbcB* 15, *hspR* 4,  $\Delta(lac-proAB)$ , [F', *traD* 36, *proAB*, *lacI*<sup>q</sup>Z,  $\Delta$ M15])(49), and BW313(*dut*, *ung*, *thi*-1, *relA*, *spo* T1/F'lys A). BW313 was a gift from Dr. Kagamiyama, Medical College of Osaka. A host for pKGS00 plasmids and M13 phages was *E. coli* JM109 (*rec A* 1, *end A* 1, *gyrA* 96, *thi*, *hsdR* 17, *supE* 44, *relA* 1,  $\lambda^{-1}$ ,  $\Delta(lac-proAB)$ , [F', *traD* 36, *pro AB*, *lacI*<sup>q</sup>Z  $\Delta$ M15])(49).

**Restriction Enzymes and Chemicals** Restriction enzymes, T<sub>4</sub>DNA ligase, kinase, and Klenow fragment of DNA polymerase I were provided by TAKARA SHUZO CO.,LTD(Kyoto). Restriction enzyme *Hga* I was purchased from New England Biolabs (Beverly). Oligonucleotides were synthesized by the solid phase phosphoramidite method(61)

and purified by high-performance liquid chromatography on Finepak SIL C<sub>18</sub>, (JASCO) or TSK-GEL DEAE-2SW, (TOYO SODA). Purified oligonucleotides were phosphorylated at 5' end with ATP and T<sub>4</sub> DNA polynucleotide kinase.

Chemicals used in this experiment were the purest grades commercially available.

**Construction of Mutagenesis Vectors** A 1817 base pairs *Bam* HI fragment encoding the entire *gsh* II gene was isolated from plasmid pGS400 and inserted into phage M13mp8w to give M13mp8w-*gsh*II (FIG. III-1). This derivative was used as a single strand DNA template for mutagenesis of cysteine residues at 122, 195 and 289. DNA template for mutagenesis of cysteine residue at 289 was an M13mp18 derivative containing a 449 base pairs *Kpn* I/*Bam* HI small fragment of the *gsh* II gene.

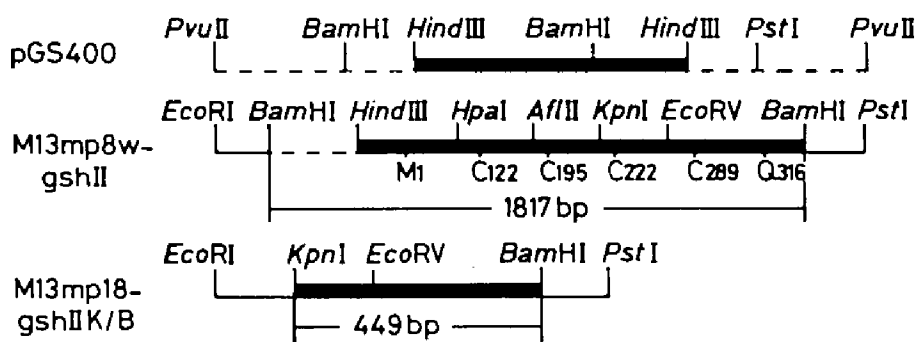


FIG. III-1 Partial restriction maps of plasmid and phages. pGS400 is a pBR325(dotted line) derivative containing a 2.6 kilobase pairs *Hind* III fragment of *gsh* II gene(solid line). The internal *Bam* HI fragment (1817 base pairs) of pGS400 was ligated into the *Bam* HI polylinker site of M13mp8w to yield M13mp8w-*gsh*II. The *Kpn* I and *Bam* HI fragment (449 base pairs) of *gsh* II gene was ligated into the *Kpn* I/*Bam* HI polylinker site of M13mp18 to yield M13mp18-*gsh*IIK/B.

**Site-Directed Mutagenesis by use of Uracil Containing Single-Stranded DNA** Oligonucleotide site-directed mutagenesis was performed by the method using uracil-containing single-stranded DNA as template (62). Single-stranded DNA template containing uracil was prepared as follows (63). BW313 cells were grown overnight in 4 ml YT medium supplemented with 20  $\mu$ g/ml thymidine and 100  $\mu$ g/ml deoxyadenosine at 37 °C with vigorous shaking. Two ml of the cultured cells were centrifuged (15 min at 2000  $\times$  g), washed with YT medium, resuspended in 100 ml of fresh 2  $\times$  YT medium pre-warmed to 37 °C, containing 0.25  $\mu$ g/ml uridine, and shaken for 5 min. To this solution, M13mp8w-gshII phage were added at a multiplicity of infection of 5 and cultured for 5 hours at 37 °C. Phage particles were collected and M13 single-stranded DNA template containing uracil was isolated (64). One pmole of the M13 DNA template was annealed with 5 pmole of mismatched oligonucleotide in 20  $\mu$ l of Solution A (20 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, and 1 mM dithiothreitol, pH 7.5). After primer extension and ligation, the DNA was used to transform competent JM105 cells. M13 replicative form DNAs carrying the desired mutation were selected as follows. Each oligonucleotide used for the cysteine to alanine mutation was designed to create a new, appropriate site of restriction cleavage (or designed to abolish an existing site of restriction cleavage) adjacent to the amino acid replacement site (Table III-I). These sites of restriction cleavage were used for screening of M13 DNAs for the desired mutations. Since the mutation of Cys-289 to Ala-289 does not introduce a new site of restriction cleavage for selection, a codon for Cys-289, TGT, was first mutated to TGA which creates an *Eco*RI site. This mutant was selected by screening with *Eco*RI. Then a codon TGA was changed to a codon of Ala by abolishing an *Eco*RI site. This mutant became silent to *Eco*RI.

Short fragment containing the mutation site was excised from the alanine mutant of M13mp8w-gshII DNA with two appropriate restriction enzymes, *Hpa*I/*Afl*II fragment for the Ala122 mutant, *Afl*II/*Kpn*I for the Ala195 mutant, and *Kpn*I/*Bam*HI for the Ala289 mutants (Fig. III-1). This small fragment was subcloned into M13mp18 or M13mp19 at their cloning site for sequence analysis by dideoxy sequencing method (65).

TABLE III-I

Nucleotide sequences of synthetic oligonucleotide primers used for mutagenesis.

Codon No.	DNA sequence change	Synthetic oligonucleotides <sup>a</sup>	Enzyme for screening
122	TGT → GCT	5'-TCTCGTTAG <b>CGT</b> CGCGCA-3'	<i>HgaI</i>
195	TGC → GCC	5'-GCC <b>CCA</b> TGGCGTAGCGAG-3'	<i>NcoI</i>
222	TGC → GCC	5'-GCCCCAGGG <b>CGT</b> ACGGTA-3'	<i>Spl I</i>
289	TGT → TGA	5'-AATCTCACGAATTCAGGTTGGGCTG-3'	<i>EcoRI</i>
289	TGA → GCT	5'-AATCTCACGAATAGCGGTTGGGCTG-3'	<i>EcoRI</i>

<sup>a</sup>Nucleotide substitutions are indicated with boldface and restriction sites created by mutation are underlined.

After verified by the sequence analysis, a fragment containing the desired mutation was isolated on 5% polyacrylamide gel electrophoresis and transferred into overexpression vector pKK223-3.

**Expression of Wild and Mutant *gsh II* Genes** *E. coli* JM109 cells transformed with pKGS00 or one of the mutant plasmids were grown in 5 ml LB medium supplemented with 50 µg/ml ampicillin at 37°C with reciprocal shaking. At log phase (about  $A_{600} = 0.7 \text{ cm}^{-1}$ ), IPTG was added to a final concentration of 1 mM (44). After 17 hour culture, the cells were harvested by centrifugation at  $10,000 \times g$  for 10 min, suspended in 2 ml of 20 mM Tris-HCl buffer, pH 7.3, and sonicated. The suspension was centrifuged at  $15,000 \times g$  for 20 min to remove cellular debris and stored at -20 °C. Total concentration of proteins in a cell-free extract was determined by the method of Lowry (19), with bovine serum albumin as standard.

***Polyacrylamide Gel Electrophoresis*** Cell-free extracts were analyzed by 10% SDS- and 7.5% native-polyacrylamide gel electrophoresis according to the methods of Laemmli and Davis, respectively (21, 66). Authentic glutathione synthetase was a preparation purified from *E. coli* C600 (50).

***Determination of Glutathione Synthetase Activity*** Protein concentration of the glutathione synthetase was determined from  $A_{280}^{1\%}=9.02\text{ cm}^{-1}$  in 50 mM potassium phosphate buffer, pH 7.0 (50). The enzyme solution, 5  $\mu\text{l}$ , was added to an assay medium composed of 5 mM  $\gamma\text{-Glu-Cys}$ , 15 mM Gly, 10 mM ATP, 10 mM  $\text{MgSO}_4$ , and 100 mM Tris-HCl buffer, pH 7.5, in a total volume of 0.1 ml. Glutathione was determined as the amount of  $\text{TNB}^{2-}$  which was formed by thiolysis of DTNB with glutathione in the presence of NADPH and glutathione reductase (17). One unit of enzyme was defined as the amount that catalyzed the formation of 1  $\mu\text{mole}$  of glutathione per hour at 37 °C.

***DTNB Titration of the Glutathione Synthetase*** Titration of sulfhydryl groups with DTNB was carried out according to the method of Ellman (67). Concentration of  $\text{TNB}^{2-}$  was determined from molar extinction coefficient value, which was experimentally determined as 13,700  $\text{cm}^{-1}$  at 412 nm. To determine the residual activities of the enzyme during the titration, 5  $\mu\text{l}$  aliquots of the reaction mixture were added into the enzyme assay medium at intervals of 2 to 20 minutes.

### III-3 RESULTS

#### ***Construction and Expression of Ala Mutant Glutathione Synthetase Genes on Plasmid pKGS00, derived from pKK223-3***

The author constructed seven mutant enzymes in which one or more cysteine residues were replaced with alanine residues. Ala122, Ala195, Ala222, and Ala289 are mutants containing a single replacement. In

Ala122 mutant, for example, Cys-122 residue is replaced with Ala-122. Two mutants, Ala122/195 and Ala222/289, are those containing replacement at two cysteine residues. A mutant in which all four cysteine residues are replaced is Cys-free mutant.

Ala122, Ala195, Ala222, and Ala289 mutants were made by site-directed mutagenesis using synthetic oligonucleotides of between 17 and 25 nucleotides in length, introducing either a single or double nucleotide substitution. Each mutation either introduced a new restriction site into the gene or deleted an existing one, and so these changes were used as markers to select the mutants. After confirmation of mutation by restriction analysis of replicative form DNA and by dideoxy sequencing (65) of single stranded DNA, a restriction fragment containing a mutation site was excised from M13 replicative form DNA, then subcloned into pKGS00 by replacing the corresponding fragment of wild-type gene.

Mutants containing multiple replacements at two or four cysteine residues were constructed by replacing a fragment containing a mutation. For example, Ala122/195 mutant gene was constructed by 1) restriction of Ala122 mutant gene on pKGS00 plasmid with *Hinc*II/*Afl*II 2) delete of a fragment containing Cys-195 residue 3) insertion and ligation of the corresponding fragment containing Ala-195 residue restricted from Ala195 mutant gene.

*E. coli* JM109 transformed with overexpression plasmids containing one of Ala mutants of pKGS00 was cultured under the same conditions to that transformed with the wild-type pKGS00. Cell-free extracts of *E. coli* JM109 were prepared and analyzed by native polyacrylamide gel electrophoresis (Fig. III-2). They showed a thick band on the gel at the almost same mobility to that of the authentic glutathione synthetase.

***Catalytic Activities of Mutant Glutathione Synthetases*** To estimate expression levels of mutant genes, cell-free extracts were submitted to SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue R-250 and scanned by a chromatoscanner. Amounts of mutant glutathione synthetases, relative to the total protein in a cell-free extract, were about 50 % averaged over seven mutant enzymes. After correction with the relative amount of mutant

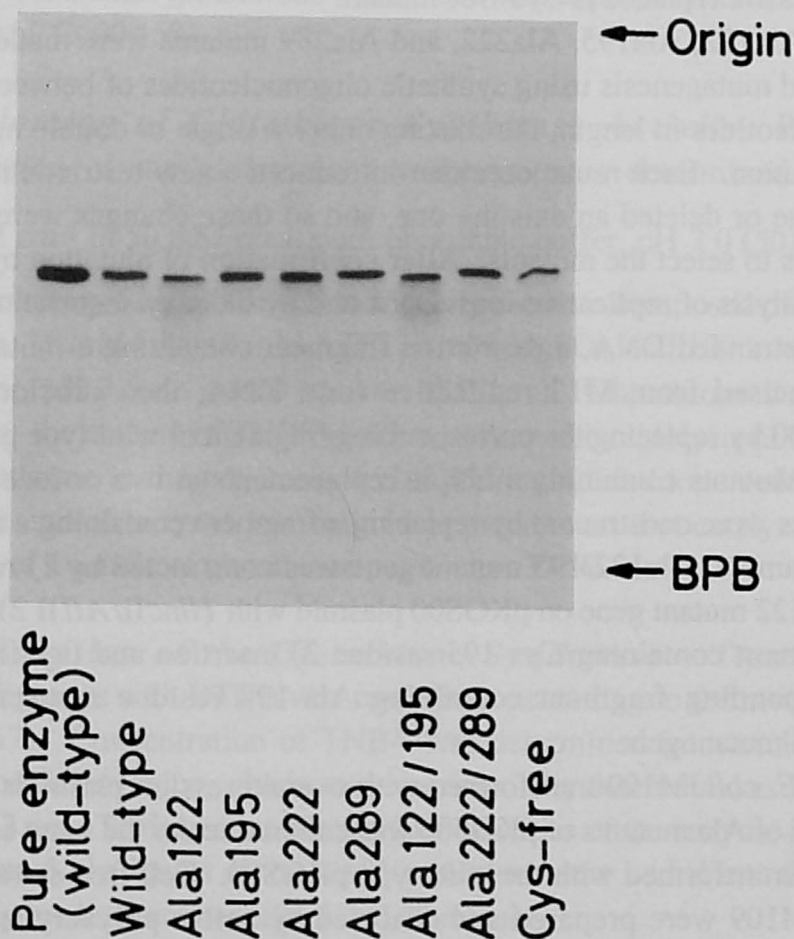


FIG. III-2. Native-polyacrylamide gel electrophoresis of the wild-type and mutant glutathione synthetases. *E. coli* JM109 containing overexpression plasmid of wild-type and mutant *gsh II* genes were grown and induced by IPTG. Sample of each cell-free extract from 15 h induced culture were electrophoresed. Pure enzyme was also Nakalai as control.

glutathione synthetase present in cell-free extract, specific activities were determined for the mutant enzymes (Table III-II). From the specific activities of Ala122, Ala195, Ala222, and Ala289 mutants, it was revealed that no critical loss in the activity was induced by the replacement of a single cysteine residue with alanine residue. In Ala222 mutant, specific activity increased to 150 % of the wild enzyme. These results, however, clearly indicated that four cysteine residues in the glutathione synthetase from *E. coli* B were not essential for the catalytic activity. Multiple replacement of cysteine residues, however, decreased enzymatic activity to 45 - 26 % of the activity of the wild-type enzyme. Specific activity of mutant enzymes, Ala122/195, Ala222/289, and Cys-free, decreased with the number of cysteine residues replaced. These results suggest that in the glutathione synthetase four cysteine residues, not a particular cysteine residue, evenly contribute to maintain its catalytic activity.

TABLE III-II  
Enzyme activities of the wild-type and mutant glutathione synthetases.

	Apparent specific activity (unit <sup>a</sup> /mg)	Expression level (%)	Specific activity (unit <sup>a</sup> /mg GSH-II <sup>b</sup> )	Relative activity <sup>c</sup>
Wild-type	22.0	53	42	1.0
Ala122	20.8	47	44	1.0
Ala195	21.2	51	42	1.0
Ala222	31.2	51	62	1.5
Ala289	19.3	50	39	0.93
Ala122/195	11.0	59	19	0.45
Ala222/289	8.18	61	13	0.31
Cys-free	3.40	31	11	0.26

<sup>a</sup> 1 unit=1  $\mu$ mol GSH/min

<sup>b</sup> mg GSH-II=[Protein amount (mg)]  $\times$  [Expression level (%)/100]

<sup>c</sup> The value for the specific activity of the wild-type enzyme has been assigned as 1.0, and the values for the mutants are relative to that of the wild-type enzyme.



***Titration of The Wild-Type Glutathione Synthetase with DTNB***

A solution of the purified glutathione synthetase (0.308mg protein in 0.93ml 0.1M phosphate buffer, pH 7.0 and 0.1mM EDTA) was placed in a spectrophotometer cell and kept at 25 °C. To this solution, 10mM DTNB 20  $\mu$ l was added and mixed rapidly. Concentration of DTNB (200  $\mu$ M) in the reaction mixture was 5.4-fold excess of the total concentration of sulfhydryl groups (37.2  $\mu$ M sulfhydryl group). Concentration of  $\text{TNB}^{2-}$  ion, which is released when DTNB reacts with sulfhydryl groups, increased rapidly in the first 10 min, then remained almost constant through 60 min. From the concentration of  $\text{TNB}^{2-}$  released for 60 min, the number of sulfhydryl groups titrated with DTNB was calculated and plotted against the time of reaction (Fig. III-3). About one mole of DTNB reacted with one mole of subunit of the glutathione synthetase; that is, among four sulfhydryl groups present in a subunit, only one sulfhydryl group reacted with DTNB. Time course of the loss of enzyme activity corresponded to the release of  $\text{TNB}^{2-}$ . Glutathione synthetase activity was rapidly decreased for the first 10 min then completely lost at 60 min by the reaction with DTNB. From this clear relation between the quantity of DTNB reacted and the degree of inhibition, it was shown that modification of one sulfhydryl group per subunit by DTNB resulted in the complete loss of the catalytic activity of the wild-type glutathione synthetase.

***Effects of Ligands on Inactivation by DTNB*** The wild-type enzyme (0.054 mg of enzyme protein in 1 ml 0.1 M phosphate buffer, pH 7.0 containing 20 mM  $\text{MgSO}_4$ ) was incubated with 10 mM each ligand and 0.1 mM DTNB at 25 °C. The enzyme was protected from inactivation by  $\gamma$ -glutamyl-L- $\alpha$ -aminobutyrate ( $\gamma$ -Glu-Abu) and partially protected by ATP.  $\gamma$ -Glu-Abu is an analogue of  $\gamma$ -Glu-Cys and significantly active as  $\gamma$ -Glu-Cys (6)(Fig. III-4). It was indicated that the modified cysteine was located at or near the active site of glutathione synthetase from *E. coli*.

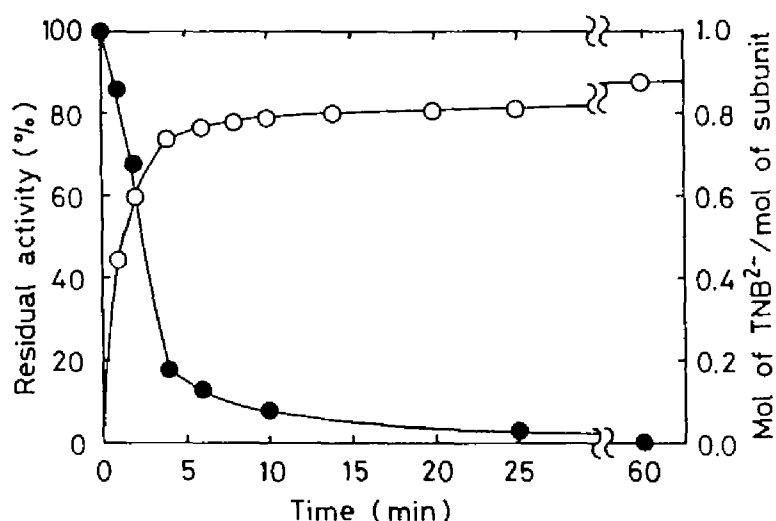


FIG. III-3. Kinetics of DTNB modification of the wild-type glutathione synthetase. Enzyme was incubated at 0.33 mg/ml with 0.2 mM DTNB in 0.1 M phosphate buffer (pH 7.0) at 25 °C. Increase in absorbance at 412 nm was recorded and aliquots (5  $\mu$ l) were withdrawn for determination of enzyme activity at appropriate time intervals. Residual activity, ●; the number of moles released TNB<sup>2-</sup>/ moles subunit, ○.

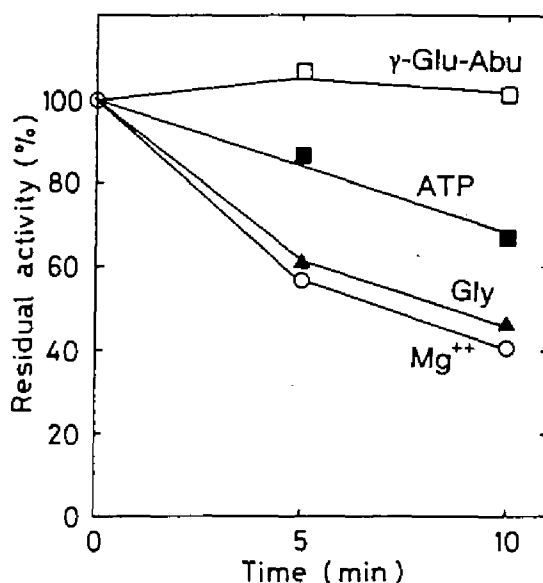


FIG. III-4. Effects of ligands on Inactivation by DTNB. The wild-type enzyme (0.054 mg/ml of glutathione synthetase protein) was incubated with 10 mM each ligand, 20 mM MgSO<sub>4</sub>, and 0.1 mM DTNB in 0.1 M phosphate buffer (pH 7.0) at 25 °C. Residual activities were determined at 5 and 10 min and shown as percent ratio to the activity at 0 min. Symbols associated with each experimental point were defined within the figure.

***Inactivation of Mutant Glutathione Synthetases by DTNB***

Cell-free extracts containing Ala122, Ala195, Ala222, and Ala122/195 mutant enzymes were inactivated at the wild-type rate in the first 10 min (Table III-III). Under the same experimental conditions, cell-free extracts containing Ala289, Ala222/289, and Cys-free mutant enzymes still retained their initial catalytic activity after 60 min of incubation. These three mutant enzymes are insensitive to DTNB and lack a sulfhydryl group at the residue 289. With purified Ala289 mutant enzyme, only 0.07 mole per subunit of  $\text{TNB}^{2-}$  ion was observed after 60 min incubation with DTNB; that is, no DTNB reacted with Ala289 mutant.

These results clearly show that Cys-289 is the only amino-acid residue reactive with DTNB in the glutathione synthetase and that modification of Cys-289 residue with DTNB results in complete loss of the catalytic activity.

**TABLE III-III**

Inactivation of the wild-type and mutant glutathione synthetases by DTNB.

Each cell-free extract was incubated at 0.1 mg/ml of glutathione synthetase protein with 0.1 mM DTNB in 0.1 M phosphate buffer (pH 7.0) at 25 °C for 10 min. Residual activity was determined under the standard assay conditions, and shown as percent ratio to the activity at 0 min.

Type of enzyme	Residual activity (%)
Wild-type	10
Ala122	6
Ala195	14
Ala222	10
Ala289	100
Ala122/195	9
Ala222/289	96
Cys-free	115

**Inactivation of Mutant Enzymes by NTCB** Cell-free extracts containing mutant enzymes (0.1 mg enzyme protein in 1 ml of 0.1 M phosphate buffer pH 7.0) were incubated with 0.1 mM NTCB at 25 °C for 60 min. While cell-free extracts of Ala122, Ala195, Ala222, and Ala122/195 were inactivated at the same rate to the wild-type enzyme, those of Ala289, Ala222/289, and Cys-free mutants enzymes retained their catalytic activity (Fig. III-5). The latter three mutant enzymes have a replacement at Cys-289 residue with Ala-289. Thus, the target of NTCB modification was proved to be the sulfhydryl group of Cys-289 residue.

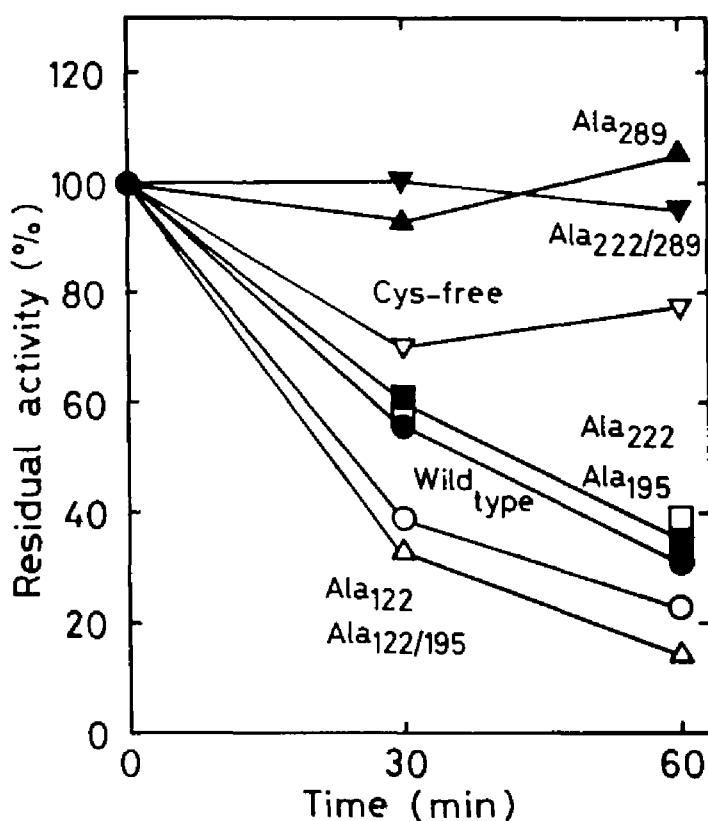


FIG. III-5. NTCB inactivation kinetics of the wild-type and mutant glutathione synthetases. Each cell-free extract (0.1 mg/ml of glutathione synthetase protein) was incubated with 0.1 mM NTCB in 0.1 M phosphate buffer (pH 7.0) at 25°C. Residual activities were determined at 30 and 60 min and shown as percent ratio to the activity at 0 min. Symbols associated with each experimental point were defined within the figure.

**Inactivation of Mutant Enzymes by NEM** Cell-free extract of each mutant enzyme (0.1 mg enzyme protein in 1 ml of 0.1 M phosphate buffer pH 7.0) was incubated with 0.3 mM NEM at 25 °C. After 10 min incubation, the wild-type enzyme was inactivated only to 60 % of the initial activity. Ala195 mutant was inactivated at the wild-type rate. These findings indicate that NEM is not so potent an inhibitor as DTNB and NTCB of the wild-type glutathione synthetase. However, Ala122 and Ala122/195 mutant enzymes were inactivated by NEM to about 10 % of the initial activity (Fig. III-6). NEM inactivated these two mutants at almost the same rate as DTNB. On the other hand, NEM failed to inactivate Ala289, Ala222/289, and Cys-free mutant enzymes, in which Cys-289 was replaced with Ala-289. From these results, NEM also modifies the sulfhydryl group of Cys-289 residue.

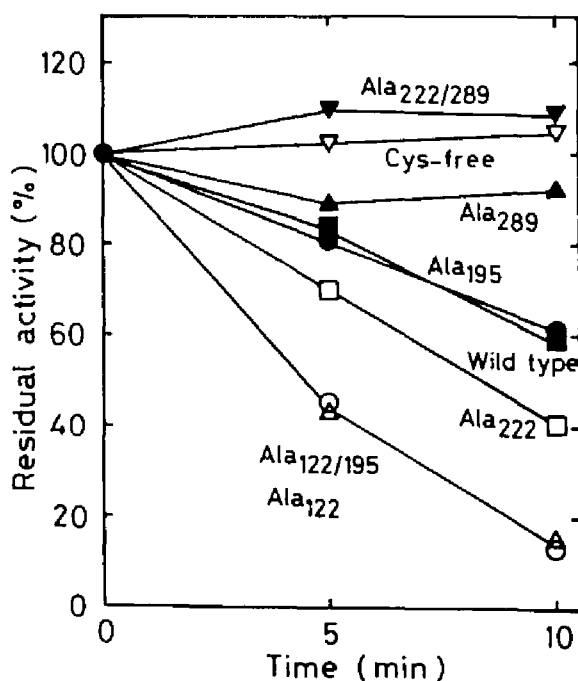


FIG. III-6. NEM inactivation kinetics of the wild-type and mutant glutathione synthetases. Each cell-free extract (0.1 mg/ml of glutathione synthetase protein) was incubated with 0.3 mM NEM in 0.1 M phosphate buffer (pH 7.0) at 25°C. Residual activities were determined at 5 and 10 min and shown as percent ratio to the activity at 0 min. Symbols associated with each experimental point were defined within the figure.

**Inactivation of Mutant Enzymes by pCMB** Cell-free extracts of each mutant enzyme (0.1 mg enzyme protein in 1 ml of 0.1 M phosphate buffer pH 7.0) were incubated with 0.1 mM pCMB at 25 °C. Ala122, Ala222, Ala289, and Ala222/289 mutant enzymes were inactivated at the same rate as the wild-type enzyme (Fig. III-7). Unlike DTNB, NTCB, and NEM, pCMB inactivated mutants containing alanine in place of Cys-289. Thus, the reactive site of pCMB is not the sulfhydryl group of Cys-289. Ala195 mutant enzyme was less inactivated. Mutant enzymes, Ala122/195 and Cys-free were not inactivated by pCMB. Accordingly, pCMB reacts with the Cys-195 residue of the glutathione synthetase.

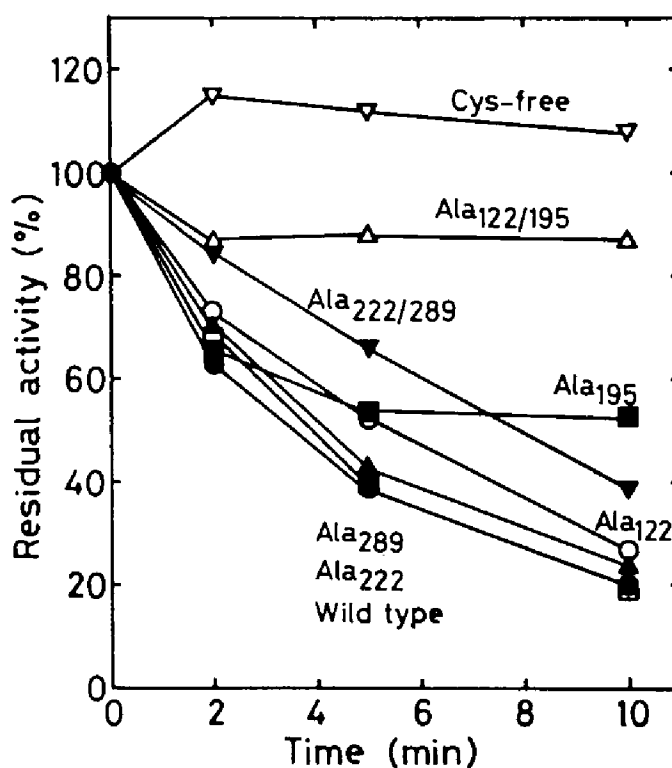


FIG. III-7. pCMB inactivation kinetics of the wild-type and mutant glutathione synthetases. Each cell-free extract (0.1 mg/ml of glutathione synthetase protein) was incubated with 0.1 mM pCMB in 0.1 M phosphate buffer (pH 7.0) at 25°C. Residual activities were determined at 2, 5, and 10 min and shown as percent ratio to the activity at 0 min: Symbols associated with each experimental point were defined within the figure.

### III-4 DISCUSSION

The author constructed seven Cys → Ala mutants of the *E. coli* B glutathione synthetase and analyzed effects of the mutations on catalytic activity and on inactivation by sulfhydryl reagents. When only one single cysteine residue in the *E. coli* B enzyme is replaced with alanine, each of four possible mutant enzymes Ala122, Ala195, Ala222, and Ala289 retained almost the same catalytic activity to the wild-type enzyme. This clearly shows that any one of the four sulfhydryl groups is not essential for the catalytic activity of the *E. coli* B glutathione synthetase.

Then, the inactivation of the enzyme by chemical modification of Cys-289 with sulfhydryl reagents is not due to the loss of chemical reactivity of the sulfhydryl group of Cys-289. While the mutation of Cys-289 to Ala-289 reduced the volume of the amino-acid side chain at residue 289 from a sulfhydryl group to a less bulky hydrogen atom, chemical modification introduces much bulkier group on the sulfhydryl group of Cys-289 and increased the volume. The wild-type enzyme was not inactivated by 0.1 mM DTNB in the presence of a substrate analogue, 10 mM  $\gamma$ -glutamyl-L- $\alpha$ -aminobutyrate. Presumably, Cys-289 of the *E. coli* B glutathione synthetase is spatially close to substrate binding at the catalytic site. Chemical modification of Cys-289 would inhibit the access or binding of a substrate to catalytic site. Profy and Schimmel (68) constructed and studied about several Cys → Ala mutants of glycyl-tRNA synthetase. Very similar to our present results, they found that a cysteine residue presumed essential from the inhibition experiments using NEM was not required for the catalysis of glycyl-tRNA synthetase. They also proposed that the inactivation of glycyl-tRNA synthetase by NEM was due to steric or conformational effects of the alkylated cysteine side chain by NEM.

In addition, the author observed some perturbations in physico-chemical properties on the *E. coli* B enzyme induced by the mutations of cysteines at residues 122 and 222. These two cysteine residues were involved neither in catalytic reaction nor inactivation by three sulfhydryl reagents, but replacement of one of the two cysteines with alanine affected the reactivity of catalytic and inactivation reactions. The replacement of

Cys-122 with Ala-122 enhanced the rate of inactivation with NTCB and NEM; that is, this replacement increased the reactivity of Cys-289 with NTCB and NEM. Mutation to Ala222 increased in both the catalytic activity and the reactivity of Cys-289 with NEM. Further, on native polyacrylamide gel electrophoresis, mutants Ala122 and Ala122/195 proteins were slightly different from those of the wild-type and the other mutants. Cysteines 122 and 222, not accessible by three sulfhydryl reagents, are probably located in inside of the enzyme. Any changes in volume of amino-acid side chains in this region are expected to induce some perturbations in tertiary structure and/or charge distributions. These perturbations were cumulative when more than two cysteine residues are replaced with alanine residues. Catalytic activities of Ala122/195, Ala222/289, and Cys-free mutants dropped to 45 - 26 % of the wild-type enzyme.

In conclusion, the present experiments unambiguously showed that four sulfhydryl reagents strictly differentiate between Cys-289 and Cys-195 residues among four cysteine residues present in the *E. coli* B glutathione synthetase. When site-directed mutagenesis of amino-acid residues is used in combination with various protein-modifying reagents, mutagenesis gives us precise informations about the mutual roles of the residues to maintain enzyme activity.



## CHAPTER IV

### Crystallization and Preliminary X-Ray Studies of Glutathione Synthetase from *Escherichia coli* B

#### IV-1 INTRODUCTION

X-ray crystallography showed us three-dimensional picture of several enzyme protein structures at atomic resolution. On the other hand, small angle X-ray scattering is a technique well suited for the determination of the overall size and shape of a protein in solution and for the characterization of structural rearrangements that occur as a consequence of ligand binding. Furthermore, when atomic coordinates of a protein are available, scattering profiles computed from high resolution models based on the coordinates can be compared to experimental profiles to identify candidate solution structures. Comparisons of crystal and solution structures using small angle X-ray scattering have been reported for a number of enzymes, such as hen egg white lysozyme (69), yeast phosphoglycerate kinase (70), and horse liver alcohol dehydrogenase (71) and so forth. However, crystallographic analysis needs an appropriate protein crystals in substantial quantity, and consumes a large amount of purified protein for prediction of crystallization conditions. As described in Chapter II, the author already constructed overexpression plasmid pKGS00, a derivative of pKK223-3 in which the *gsh* II gene is inserted behind the *tac* promoter and induction of *E. coli* JM109 cells containing pKGS00 with IPTG resulted in production of the enzyme as 53 % of soluble protein. From the resulting cell extract, glutathione synthetase was readily purified only 2 steps procedure as large amount as to need in crystallization experiments.

In this Chapter, the author describes the crystallization, preliminary crystallographic studies on the glutathione synthetase from *E. coli* B by X-ray diffraction, and also a small angle X-ray scattering measurement on the enzyme in aqueous solution.

## IV-2 EXPERIMENTAL PROCEDURES

**Materials** Specially prepared ammonium sulfate, and IPTG were purchased from NACALAI TESQUE, INC.(Kyoto), DEAE-Cellulofine A800 from Chisso co. (Minamata). *E. coli* JM109 containing pKGS00 was described before (72; Chapter II).

**Glutathione Synthetase Activity** Protein concentration of the glutathione synthetase was determined from  $A_{280}^{1\%} = 9.02 \text{ cm}^{-1}$  in 50 mM potassium phosphate buffer, pH 7.0 (50). The enzyme solution, 5  $\mu$ l was added to an assay medium composed of 5 mM  $\gamma$ -Glu-Cys, 15 mM Gly, 10 mM  $\text{MgSO}_4$ , and 100 mM Tris-HCl buffer, pH 7.5, in a total volume of 0.1 ml. GSH was determined as the amount of  $\text{TNB}^{2-}$  which was formed by thiolysis of DTNB with GSH in the presence of NADPH and glutathione reductase (17). One unit of enzyme was defined as the amount that catalyzed the formation of 1  $\mu$ mole GSH per min at 37 °C.

**Cell Growth and Purification of the Enzyme** The *E. coli* JM109 cells containing pKGS00 were grown in the 500 ml of LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 50  $\mu$ g/ml ampicillin, at pH 7.5, 37 °C with reciprocal shaking. At log phase ( $A_{600} = 0.7 \text{ cm}^{-1}$ , about 2.2 h), IPTG was added at the final concentration of 1 mM. After 17 hours, the cells were harvested by centrifugation ( 2.8 g wet weight ), suspended in 20 mM Tris-HCl buffer (pH 7.3) containing 5 mM  $\text{MgCl}_2$ , and disrupted by sonication for 5 min. Cellular debris was removed by centrifugation. Protein concentration of the extract (380 mg) was adjusted at 10 mg/ml, then finely powdered ammonium sulfate was

added to the extract as 35% saturation. After standing for 2 hours at 0 °C, microcrystals were appeared and collected by centrifugation at  $10,000 \times g$  for 15 min at 0 °C. These microcrystals were dissolved in 20 mM Tris HCl, pH 7.3 containing 5 mM  $MgCl_2$  and dialyzed against same buffer containing 5 mM  $MgCl_2$  and 0.1 M NaCl. The dialyzate was applied to a DEAE-Cellulofine A-800 column ( $2.4 \times 33$  cm) equilibrated with the same buffer. After washing the column with 300 ml of the same buffer, the enzyme was eluted with 0.15 M NaCl in the buffer. The active fractions (151 mg protein) were collected and concentrated by ultrafiltration and dialyzed against 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM  $MgCl_2$ . The purity of the enzyme was estimated by electrophoresis in the presence of sodium dodecyl sulfate (21).

**Crystallization** Crystals were grown by vapor diffusion equilibration of 20  $\mu$ l drops hanging from siliconized microscope cover slips inverted on disposable plastic tissue culture plates (73) and by microdialysis methods using a 100  $\mu$ l siliconized cell, called "button"(Fig. IV-1) which was originally developed by Butler and Taylor (74). Protein solutions at a concentration of 5-30 mg/ml were prepared in the presence of 10-40 % saturated ammonium sulfate as precipitating agents in 50 mM potassium phosphate buffer ,containing 5 mM  $MgCl_2$  at pH range 5.5-6.8. The temperature was controlled to keep at 25°C.

**X-Ray Diffraction Experiments** Crystals were transferred to a 40% saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 5.5 containing 5 mM  $MgCl_2$ . Crystals were mounted in thin-walled quartz capillaries and were photographed on a precession camera (Chales Supper Co. Inc.) at 25 °C using nickel-filtrated Cu- $K\alpha$  radiation from a Rigaku RU-200 rotating anode generator operated at 40 kV and 100 mA. The crystal-to-film distance was set to 100 mm. Cell parameters were obtained using a Rigaku C-5 4-circle diffractometer at 10 °C.

**Small Angle X-Ray Scattering Experiments** The experiments was performed according to Tanaka et al (75) and Morimoto (76). The X-ray source was a  $0.4 \times 8$  mm spot on the copper anode of a Philips fine-

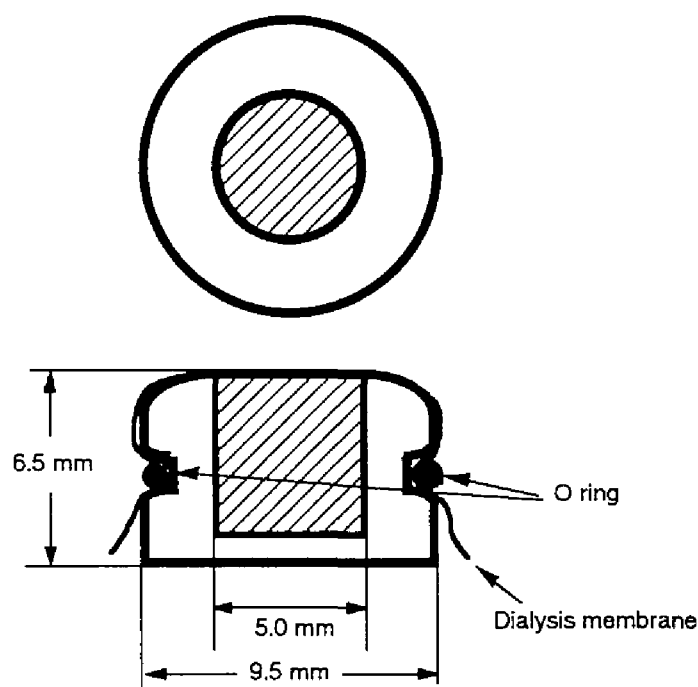


FIG. IV-1. A plastic dialysis cell, for the growth of crystals in a volume of 0.1 ml. The sample is injected in the cells and is covered by a dialysis membrane secured by a piece of O ring, then the cell is placed in the outer solution in which appropriate concentration of precipitate reagents are contained.

focus X-ray tube, which was opened with a Rigaku D9C X-ray generator run at 40 kV, 36 mA. Line focus was used to obtain sufficiently intense scattered X-rays. The spot was foreshortened to  $0.04 \times 8$  mm at a glancing angle of  $6^\circ$ . The nickel-filtered ( $10 \mu\text{m}$  in thickness) X-rays were collimated with a  $0.3 \times 10$  mm slit and then reflected and focused by a nickel-coated glass mirror through two kinds of limiting slits (77) The sample solution was sucked into a thin walled quartz capillary tube (1.0 mm diameter) by the injector through the tigon tube connected at the top of the capillary tube. The scattered X-rays were recorded on a one-dimensional position-sensitive proportional detector (delay-line type). The flowing gas (11 mm in thickness) used was a mixture of 90% argon and 10% methane. The width of the detector length was 50 mm and was divided into 512 channels on a multichannel analyzer. The sample-to-detector

distance was 343.5 mm. The scattered X-rays were accumulated for 4000 s in the range of the scattering angles from  $3.0 \times 10^{-3}$  to  $7.7 \times 10^{-2}$  rad. Each scattering intensity measurements were repeated 10 times. The X-ray path from the sample holder to the detector was evacuated to avoid air scattering. The X-ray experiments were performed at 10 °C. To avoid errors arising from interparticle interference, the scattering intensities were measured with four dilutions (3.0, 5.0, 8.0, and 12.0 mg of protein/ml in 50 mM potassium phosphate buffer, pH 6.8 containing 5 mM  $\text{MgCl}_2$ ), and no difference in the scattering profiles was observed for the four dilutions. In order to correct the background X-ray scattering, the buffer solution was also measured. The scattering intensities recorded on both side of a primary beam were averaged at equivalent points after subtracting background intensities. The center of the primary beam (zero angle) was precisely searched so as to realize the best coincidence of the scattering intensities at the equivalent points on both sides of the zero angle. The coincidences expressed by a reliability factor  $R_{sym}$  (78) were good. The independent scattering intensity,  $I(s)$ , was then obtained by the deconvolution (desmearing) for beam height effect according to the method of Glatter (79). The scattering parameter,  $s$ , is defined by  $s = 4\pi \sin \theta / \lambda$ , where  $2\theta$  is the scattering angle and  $\lambda$  the wavelength of X-ray ( $\lambda = 1.5418 \text{ \AA}$ ). The radius of gyration,  $R_g$ , and zero-angle scattering profile,  $I(0)$ , were derived from Guinier plot (80) of the scattering intensity data by a least squares method. In the course of data analysis, the distance distribution function,  $P(r)$ , was calculated by a Fourier transform of the scattering profile (81).

$$P(r) = \frac{1}{2\pi^2} \int_0^\infty I(s) \cdot sr \cdot \sin sr \cdot ds$$

The maximum particle dimension,  $D_{max}$ , was calculated from the distance,  $r$ , where  $P(r)$  finally vanishes.

### IV-3 RESULTS AND DISCUSSION

**Crystallization** Crystals of glutathione synthetase shown in Fig. IV-

2 were obtained as prisms (half-hexagonal) by vapor diffusion methods. The droplets consisted of 10  $\mu$ l of a pure enzyme solution containing 20 mg protein/ml plus 10  $\mu$ l of a 27 % saturated  $(\text{NH}_4)_2\text{SO}_4$  in 50 mM potassium phosphate buffer, pH 5.5 containing 5 mM  $\text{MgCl}_2$  and 0.04%  $\text{NaN}_3$ . These droplets were equilibrated against 1 ml of 27% saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 5.5 containing 5 mM  $\text{MgCl}_2$ . After 2 days at room temperature, the crystals have dimension up to  $0.3 \times 0.2 \times 0.1$  mm which were not large enough for X-ray diffraction studies.

More large crystals of the enzyme were obtained by the micro dialysis methods (Fig. IV-3). One-hundred  $\mu$ l of the protein solution containing 2 % glutathione synthetase, 20 % saturated  $(\text{NH}_4)_2\text{SO}_4$ , and 5 mM  $\text{MgCl}_2$  in 50 mM potassium phosphate buffer (pH 5.5) was dialyzed against 30 ml of 27 % saturated  $(\text{NH}_4)_2\text{SO}_4$ , 5 mM  $\text{MgCl}_2$ , and 0.02 %  $\text{NaN}_3$  in the same buffer at 25 °C. The large crystals ( $1.2 \text{ mm} \times 1.0 \text{ mm} \times 0.4 \text{ mm}$ ) which could be subjected to X-ray diffraction studies were grown in a week.

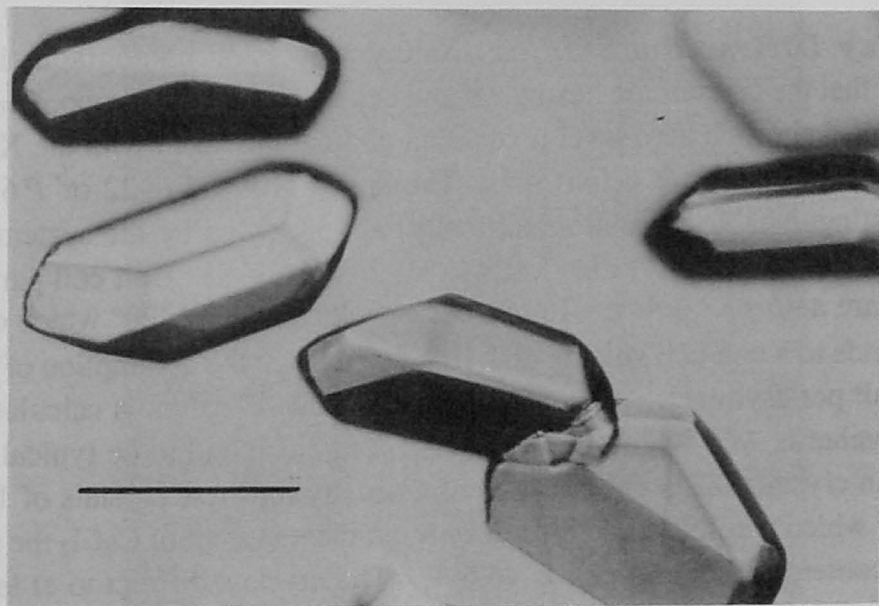


Fig. IV-2. Microphotograph of the glutathione synthetase crystals. Inner scale is 0.2 mm.

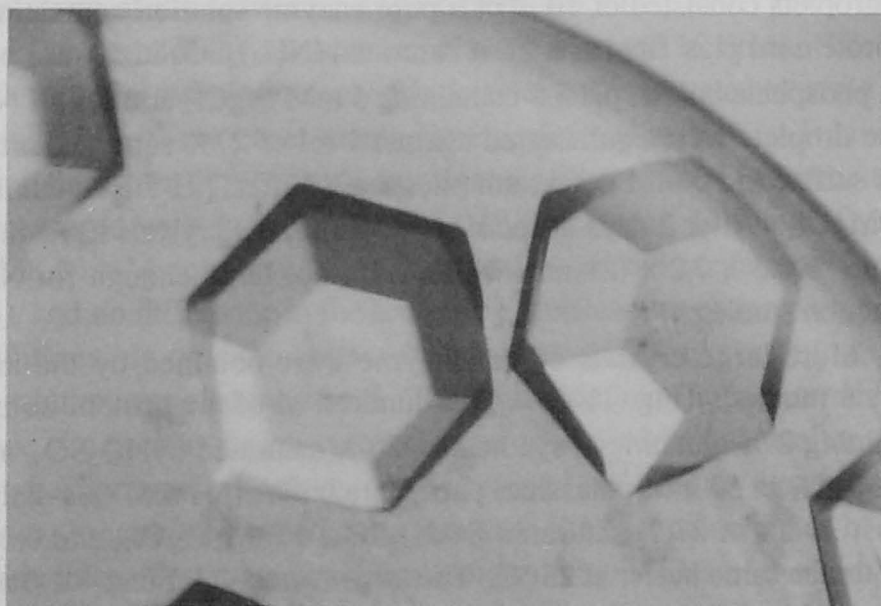


FIG. IV-3. Photograph of glutathione synthetase crystals in microdialysis cell. The large crystal is approximately  $1.2 \times 1.0 \times 0.4$  mm.

**X-Ray Diffraction Studies** X-Ray precession photographs indicated that the crystals are hexagonal and belong to the Laue group,  $6/mmm$ . Fig. IV-4 shows a zero level precession photograph taken with the X-ray beam parallel to the 6-fold axis. The space group  $P6_222$  or  $P6_422$  (indistinguishable crystallographically) are specified by the systematic absence of reflections  $l=3n+1$  and  $l=3n+2$  (Fig. IV-5). Unit cell parameters are  $a=b=88.0$  Å,  $c=164.2$  Å,  $\alpha=\beta=90^\circ$ , and  $\gamma=120^\circ$  which corresponds to a unit cell volume of  $1.10 \times 10^6$  Å<sup>3</sup>. On the assumption of one subunit per asymmetric unit, a  $V_M$  value of  $2.56$  Å<sup>3</sup>/dalton is calculated. This value is well within the range of 1.6 to 3.6 found to be typical for protein crystals (82). On the basis of a density for these crystals of  $1.23$  g/cm<sup>3</sup> which was measured by a density-gradient system of CsCl<sub>2</sub> the solvent content of the unit cell is 46.6 %. The crystals diffract to at least  $2.5$  Å resolution. X-ray diffraction data collections and search for heavy-atom derivatives are in progress.

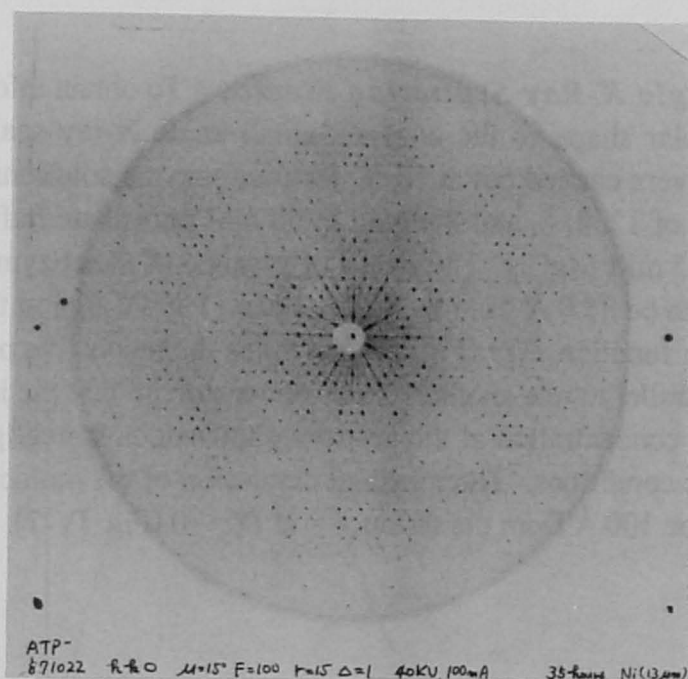


FIG. IV-4. Precession photograph of the  $hk0$  zone of the glutathione synthetase crystal. Precession angle,  $\mu=15^\circ$  (3.0 Å resolution).

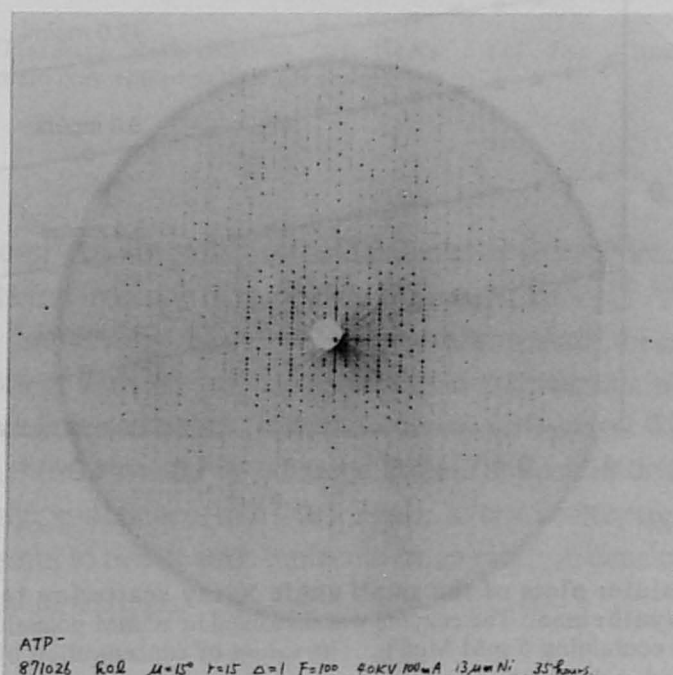


FIG. IV-5. Precession photograph of the  $h0l$  zone of the glutathione synthetase crystal. Precession angle,  $\mu=15^\circ$  (3.0 Å resolution).



**Small Angle X-Ray Scattering Studies** To obtain information on the molecular shape of the enzyme, small angle X-ray scattering experiments were carried out at 10 °C for four enzyme solutions with concentrations of 12, 8, 5, and 3 mg/ml in 50 mM phosphate buffer, pH 6.8 containing 5 mM  $\text{MgCl}_2$ . The radius of gyration of the enzyme molecule was found to be 35.0 Å from the Guinier plot (Fig. IV-6) and the distance distribution function  $P(r)$  (Fig. IV-7). Since the respective plots in Fig. VI-6 are parallel to one another, it can be considered that the influence of the protein concentration at the scattering intensities is negligibly small under these conditions. The maximal dimension of the molecule was estimated to be 100 Å from the distance  $r$  at  $P(r)=0$  (Fig. IV-7).

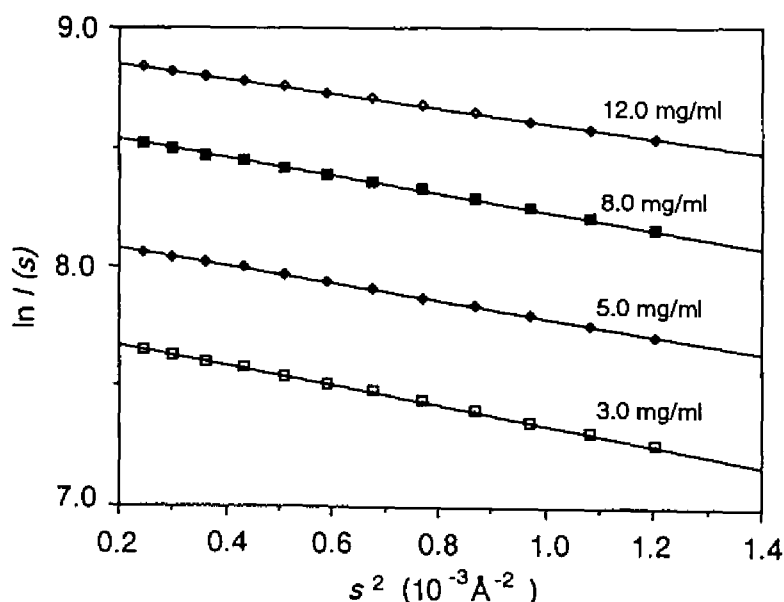


FIG. IV-6. Guinier plots of the small angle X-ray scattering intensities of glutathione synthetase. The enzyme was dissolved in 50 mM potassium phosphate buffer, pH 6.8 containing 5 mM  $\text{MgCl}_2$ . The values of concentration of the enzyme were defined within the figure.

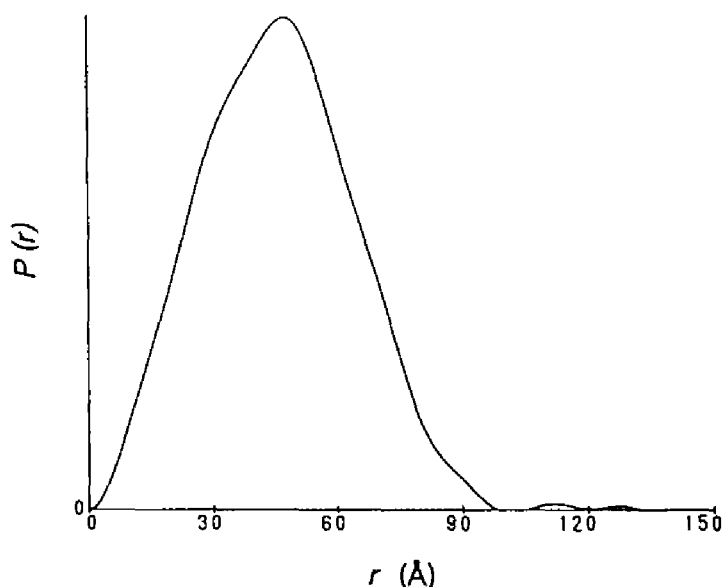


FIG. IV-7. Distance distribution functions  $P(r)$  for glutathione synthetase. Protein concentration was 3.0 mg/ml.

The volume of the enzyme molecule calculated from Porod's invariant and the scattered intensity at zero angle (83) was  $2.28 \times 10^5 \text{ \AA}^3$ , which was larger than the  $1.93 \times 10^5 \text{ \AA}^3$  in the crystalline state, because of the hydration effect with the enzyme molecule in the aqueous solution. The molecular weight estimated from the volume (84) was 140,000, which agreed with those obtained by other methods (7). Such information on the molecular shape obtained by small angle X-ray scattering techniques should be useful to isolate each molecule on an electron density map on the crystal structure analysis.

## GENERAL CONCLUSION

Glutathione synthetase (EC 6.3.2.3) from *Escherichia coli* B showed amino acid sequence similarity with mammalian and bacterial dihydrofolate reductases over 40 residues, although these two enzymes are different in their reaction mechanisms and ligand requirements. Thus the effects of ligands of dihydrofolate reductase on the reaction of *E. coli* B glutathione synthetase were examined to find resemblances in catalytic function to dihydrofolate reductase. Consequently, it was found that the *E. coli* B enzyme was potently inhibited by 7,8-dihydrofolate, methotrexate, and trimethoprim. From more detailed studies, methotrexate was proved to bind to an ATP binding site of the *E. coli* B enzyme with  $K_i$  value of 0.1 mM. In addition, the similar portion of the amino acid sequence in dihydrofolate reductases, which corresponded to the portion coded by exon 3 of mammalian dihydrofolate reductase genes, are known to provide a binding site of the adenosine diphosphate moiety of NADPH in the crystal structure of dihydrofolate reductase. These analyses would indicate that the similar portion of the amino acid sequence of the *E. coli* B enzyme provides the ATP binding site. This is the first experimental evidence that amino acid sequences related by sequence similarity conserve functional similarity even in enzymes being different in their catalytic mechanisms.

The overexpression plasmid, pKGS00, in which the glutathione synthetase gene from *E. coli* B (*gsh* II) was controlled by *tac* promoter and *rrn* B terminator, was constructed. The kinetics and extent of induction in *E. coli* JM109 showed that after 17 hr of induction (total 19 hr culture), the glutathione synthetase in active form constituted about 53 % of the total soluble protein of *E. coli* cells. The specific activity of the cell-free extract from the cells transformed with pKGS00 was 660-fold of that observed in chromosomal levels of *E. coli* JM109. From 500 ml of

culture, two purification procedures gave 130 mg of a purified enzyme preparation.

*E. coli* B glutathione synthetase is composed of four identical subunits; each subunit contains four cysteine residues (Cys-122, 195, 222, and 289). Then, the author constructed seven different mutant enzymes containing three, two, or no cysteine residues per subunit by replacement of cysteine codons with those of alanine in the *gsh* II gene using site-directed mutagenesis. Among of them, three mutant enzymes Ala289, Ala222/289, Cys-free(Ala122/195/222/289), in which cysteine at residue 289 was replaced with alanine, were not inactivated by 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), while the other four mutants retaining Cys-289 were inactivated at the wild-type rate. From these selective inactivations of mutant enzymes by DTNB, the sulfhydryl group modified by DTNB was unambiguously identified as Cys-289. In this way, Cys-289 was found to be also a target of modification with 2-nitrothiocyanobenzoate (NTCB) and N-ethylmaleimide (NEM), while Cys-195 was of p-chloromercuribenzoate (pCMB). These results suggest that both Cys-195 and Cys-289 were not essential for the activity of the glutathione synthetase, but chemical modification of either one of the two sulfhydryl groups resulted in complete loss of the activity. Replacement of Cys-122 to Ala-122 enhanced the reactivity of Cys-289 with sulfhydryl reagents.

Preliminary X-ray studies on *E. coli* B glutathione synthetase have been carried out using crystallographic and small angle scattering techniques. The glutathione synthetase from *E. coli* B was crystallized to suitable for X-ray analysis. It is the first time to succeed in the crystallization of glutathione synthetase. The crystals are hexagonal, space group  $P6_22$  or  $P6_422$  (indistinguishable crystallographically). The cell dimensions are  $a = b = 87.8 \text{ \AA}$ ,  $c = 163.9 \text{ \AA}$ ,  $\alpha = \beta = 90^\circ$ , and  $\gamma = 120^\circ$ . The enzyme is a tetramer of  $M_r 143,000$ , and the asymmetric unit contains one subunit molecule of  $M_r 35,600$ . The crystals diffracted to at least  $2.5 \text{ \AA}$  resolution. A small angle X-ray scattering study showed that the radius of gyration and the maximal dimension of the tetramer molecule in aqueous solution are  $35.0 \text{ \AA}$  and  $100 \text{ \AA}$ , respectively.

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## REFERENCES

1. Meister, A. (1974) in *The Enzymes* (Boyer, P.D. ed.) Vol X third ed., pp.688-697, Academic Press, New York
2. Meister, A (1985) in *Method in Enzymology* (Meister, A. ed.) Vol 113, pp.393-399, Academic Press, New York
3. Meister, A. (1974) in *The Enzymes* (Boyer, P.D. ed.) Vol X third ed., pp.674-687, Academic Press, New York
4. Meister, A (1985) in *Method in Enzymology* (Meister, A. ed.) Vol 113, pp.379-392, Academic Press, New York
5. Mooz, E.D. and Meister, A. *Biochemistry* 6, 1722-1734
6. Oppenheimer, L., Wellner, V.P., Griffith, O.W., and Meister, A. (1979) *J. Biol. Chem.* 254, 5184-5190
7. Gushima, H., Miya, T., Murata, K., and Kimura, A. (1983) *J. Appl. Biochem.* 5, 210-218
8. Moore, W. R., and Meister, A. (1987) *Anal. Biochem.* 161, 487-493
9. Nishimura, J. S., Dodd, E. A., and Meister, A. (1963) *J. Biol. Chem.* 238, 1179-1180
10. Nishimura, J. S., Dodd, E. A., and Meister, A. (1964) *J. Biol. Chem.* 239, 2553-2558
11. Wendel, A. & Flohe, L. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 523-530
12. Blow, D. M., Fersht, A. R., and Winter, G. (1986) *Phil. Trans. R. Soc. Lond. A* 317, 291-451
13. Ulmer, K. M. (1983) *Science* 219, 606-611
14. Gushima, H., Yasuda, S., Soeda, E., Yokota, M., Kondo, M., and Kimura, A. (1984) *Nucl. Acids Res.* 12, 9299-9307
15. Gushima, H., Miya, T., Murata, K., and Kimura, A. (1983) *J. Appl. Biochem.* 5, 43-52
16. Murata, K., Kato, J., and Kimura, A. (1978) *Agric. Biol. Chem.* 42, 2221-2228
17. Tietze, F. (1969) *Anal. Biochem.* 27, 502-522
18. Mathews, C.K., Scrimgeour, K.G., and Huennekens, F.M. (1963) in

- Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O., eds.) Vol VI, pp.364-368, Academic Press, New York
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* 193, 256-275
  20. Kupke, D.W. and Dorrier, T.E. (1978) in *Methods in Enzymology* (Hirs, C.H.W. and Timasheff, S.N., eds.) Vol XLVIII, pp.155-162, Academic Press, New York
  21. Laemmli, U.K. (1970) *Nature* 227, 680-685
  22. Wilkinson, G.N. (1961) *Biochem. J.* 80, 324
  23. Ooi, T., and Nakashima, H. (1985) in *Protein Engineering* (in Japanese) (CMC, ed.) pp.19-29, CMC, Tokyo
  24. Kanehisa, M. (1982) *Nucl. Acids Res.* 10, 183-196
  25. Goad, W.B., and Kanehisa, M. (1982) *Nucl. Acids Res.* 10, 247- 263
  26. Dayhoff, M.O., Schwartz, R.M., and Orcutt, B.C. (1978) in *Atlas of Protein Sequence and Structure* Vol. 5, Supplement 3, pp. 345-352, National Biomedical Research Foundation, Washington, D.C.
  27. Stone, S.R. and Morrison, J.F. (1986) *Biochim. Biophys. Acta* 869, 275-285
  28. Cleland, W.W. (1963) *Biochim. Biophys. Acta* 67, 104, 173, 188
  29. Cleland, W.W. (1970) in *The Enzymes* (Boyer, P.D. ed.) Vol II third ed., pp.1-65, Academic Press, New York
  30. Filman, D.J., Bolin, J.T., Matthews, D.A., and Kraut, J (1982) *J. Biol. Chem.* 257, 13663-13672
  31. Bolin, J.T., Filman, D.J., Matthews, D.A., Hamlin, R.C., and Kraut, J. (1982) *J. Biol. Chem.* 257, 13650-13662
  32. Volz, K.W., Matthews, D., Alden, R.A., Freer, S.T., Hansch, C., Kaufman, B.T., and Kraut, J. (1982) *J. Biol. Chem.* 257, 2528-2536
  33. Gilbert, W. (1978) *Nature* 271, 501
  34. Lonberg, N., and Gilbert, W. (1985) *Cell* 40, 81-90
  35. Crouse, G.F., Simonsen, C.C., McEwan, R.N., and Schimke, R.T. (1982) *J. Biol. Chem.* 257, 7887-7897
  36. Setzer, D.R., McGrogan, M., and Schimke, R.T. (1982) *J. Biol. Chem.* 257, 5143-5147
  37. Chen, M.J., Shimada, T., Moulton, A.D., Cline, A., Humphries, R.K., Maizel, J., and Nienhuis, A.W. (1984) *J. Biol. Chem.* 259, 3933-3943

38. Go, M. (1981) *Nature* **291**, 90-92
39. Go, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1964-1968
40. Rossmann, M.G., and Lilijas, A. (1974) *J. Mol. Biol.* **85**, 177-181
41. Stone, S.R. and Morrison, J.F. (1982) *Biochemistry* **21**, 3757-3765
42. Pouwels, P. H., Enger-Valk, B. E., & Brammar, W. J. (1985) in *Cloning Vectors. A Laboratory Manual*, Elsevier, Amsterdam
43. deDoer, H.A., Comstock, L.J., and Vasser, M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 21-25
44. Amann, E., Brosius, J., and Ptashne, M. (1983) *Gene* **25**, 167-178
45. Thomas, D. C., Levy, M., and Sancar, A. (1985) *J. Biol. Chem.* **260**, 9875-9883
46. Barstow, D. A., Clarke, A. R., Chia, W. N., Wigley, D., Sharman, A. F., Holbrook, J. J., Atkinson, T., and Minton, N. P. (1986) *Gene* **46**, 47-55
47. Milton, D. L., Napier, M. L., Myers, R. M., and Hardman, J. K. (1986) *J. Biol. Chem.* **261**, 16604-16615
48. Brosius, J., Dull, T. J., Sleeter, D. D., and Noller, H. F. (1981) *J. Mol. Biol.* **148**, 107-127
49. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene* **33**, 103-119
50. Kato, H., Chihara, M., Nishioka, T., Murata, K., Kimura, A., and Oda, J. (1987) *J. Biochem (Tokyo)* **101**, 207-215
51. Spackman, D.H., Stein, W.H., and Moore, S. (1958) *Anal. Chem.* **30**, 1190-1206
52. Moore, S. (1963) *J. Biol. Chem.* **238**, 235-237
53. Iserentant, D., and Fiers, W. (1980) *Gene* **9**, 1-12
54. Johnston, H. M., and Roth, J.R. (1981) *J. Mol. Biol.* **145**, 735-756
55. Schwartz, M., Roa, M., and Debarbouille, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2937-2941
56. Hall, M. N., Gabay, J., Debarbouille, M., and Schwartz, M. (1982) *Nature* **295**, 616 (1982).
57. Gheysen, D., Isereutant, D., Derom, C., and Fiers, W. (1982) *Gene* **17**, 55-63
58. Schottel, J. L., Sninsky, J. J., and Cohen, S. N. (1984) *Gene* **28**, 177-193



59. Buell, G., Schulz, M. F., Selzer, G., Chollet, A., Movva, N. R., Semon, D., Escanez, S., and Kawashima, E. (1985) *Nucleic Acids Res.* 13, 1923-1938
60. deBoer, H. A., Hui, A., Comstock, L. J., Wong, E., and Vasser, M. (1983) *DNA* 2, 231-235.
61. Sinha, N. D., Biernat, J., and Koester, H. (1983) *Tetrahedron Lett.*, 24, 5843-5846
62. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 488-492
63. Sagher, D., and Strauss, B. (1983) *Biochemistry* 22, 4518-4526
64. Messing, J. (1983) in *Methods in Enzymology* (Wu, R., Grossman, L., and Moldave, K. ed.) Vol. 101, pp. 20-78, Academic Press, New York
65. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467
66. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* 121, 404-427
67. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* 82, 70-77
68. Profy, A. T., and Schimmel, P. (1986) *J. Biol. Chem.* 261, 15474-15479
69. Krigbaum, W. R., and Kügler, F. R. (1970) *Biochemistry* 9, 1216-1223
70. Pickover, C. A., McKay, D. B., Engelman, D. M., and Steitz, T. A. (1979) *J. Biol. Chem.* 254, 11323-11329
71. Pavlov, M. Y., Sinev, M. A., Timchenko, A. A., and Ptitsyn, O. B. (1986) *Biopolymers* 25, 1385-1397
72. Kato, H., Tanaka, T., Nishioka, T., Kimura, A., and Oda, J. (1988) *J. Biol. Chem.* 263, 11646-11651
73. McPherson, A. (1982) in *Preparation and Analysis of Protein Crystals*, pp. 96-97, John Wiley & Sons., New York
74. Blundel, T. L., & Johnson, L. N. (1976) in *Protein Crystallography*, pp. 71-75, Academic Press, Orlando, FL
75. Katagiri, C., Sato, M., and Tanaka, N. (1987) *J. Biol. Chem.* 262, 15857-15861
76. Morimoto, Y. (1987) Dr. Thesis, Osaka University
77. Franks, A. (1955) *Proc. Phys. Soc. Lond. Sect. B* 68, 1054-1064
78. Blundel, T. L., & Johnson, L. N. (1976) in *Protein Crystallography*,

- pp. 318-319, Academic Press, Orlando, FL
79. Glatter, O. (1974) *J. Appl. Crystallogr.* 7, 147-153
80. Guinier, A., & Fournet, G. (1955) in *Small-Angle Scattering of X-rays*, pp. 126, John Wiley and Sons, New York
81. Pilz, I., Glatter, O., & Kratky, O. (1979) *Methods Enzymol.* 61, 148-249
82. Matthews, B. W. (1968). *J. Mol. Biol.* 33, 491-497.
83. Piltz, I. (1982) in *Small Angle X-ray Scattering* (Glatter, O., and Kratky, O., eds.) pp. 239-328, Academic Press, Inc., New York
84. Kumosinski, T. F., and Passen, H. (1982) *Arch. Biochem. Biophys.* 219, 89-100

## LIST OF PUBLICATION

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